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(57) Abstract

Methods for generating antigen-specific cytotoxic T lymphocytes (CTLs) *in vitro* are presented in conjuntion with methods for using these CTLs for therapy *in vivo*. The antigen presenting cells (APCs) used herein notably include dentritic cells pretreated with GM-CSF and IL-4. Antigen-specific CTLs were obtained by incubating CD8 preparations in the presence of APCs that bound desired peptides. IL-7 was generally added at day 1 of the incubation; IL-10 was generally added one day later and during restimulation. The invention also comprises methods for using peptide pulsed dentritic cells to identify novel MHC Class I-restricted tumor antigens and subdominant epitopes.

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PEPTIDES AND PEPTIDE-LOADED ANTIGEN PRESENTING CELLS FOR THE ACTIVATION OF CTL

This application claims priority from and is a continuation-in-part of provisional application No. 60/036,696, filed on January 31, 1997, which is incorporated by reference.

BACKGROUND OF THE INVENTION

The present invention relates to compositions and methods for preventing or treating pathological states such as viral diseases and cancer through *ex vivo* therapy. In particular, it provides methods for inducing cytotoxic T lymphocytes (CTL) using antigen presenting cells (APC) with a peptide of choice bound to selected major histocompatibility complex (MHC) molecules.

CTLs (or CD8 cells as they are also known) specifically recognize cells that bear foreign antigens, and kill them. They constitute a main line of defense against diseased states such as those caused by viral infections and tumor cells.

In contrast to antibodies, antigen must first be presented to the T cell receptors for activation to occur. CTLs recognize, not the intact foreign antigen itself, but rather 8-12 residue peptide fragments bound to MHC class I molecules and presented on the surface of antigen-presenting cells (APCs).

The presentation of antigen to T cells is mediated by the major histocompatibility complex (MHC) molecules, also referred to as the HLA (human leukocyte antigen) complex. MHC gene products are present on cell surfaces and are largely responsible for recognition of tissue transplants as "non-self".

MHC molecules are classified as either Class I, Class II or class III molecules. Class I MHC molecules are expressed on almost all nucleated cells and are recognized by CTLs. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses, such as T lymphocytes, B lymphocytes, macrophages, *etc.* Class II MHC molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of the immune response to the particular immunogenic peptide that is displayed.

In general, CTL responses are not directed against all possible determinants but only against a selected few "immunodominant" epitopes. According to the nomenclature developed by Sercarz *et al.*, *Annu. Rev. Immunol.* 11:729 (1993), an epitope is immunodominant when it is normally recognized in the course of a natural infection, or upon immunization with whole or naturally processed antigens. Subdominant epitopes mediate immune responses if 1) synthetic peptides are used as immunogens, or 2) the dominant epitopes are removed (or altered) from the whole antigen. As with dominant epitopes, the subdominant epitopes are also processed by the APC and presented as MHC complexes on the surface.

A theoretically attractive approach for the treatment of diseases as cancer, AIDS, hepatitis and other infectious disease is to activate only those CTLs that recognize diseased cells. One possible approach is to immunize a healthy individual, isolate the CTLs from this individual, and inject these cells into the diseased person. However, the MHC haplotype of the donor must be identical to that of the recipient. This is important because the CTLs of the recipient can only interact with peptides bound to one of the three to six Class I molecules present in the individual. Also, CTLs react violently with all Class I molecules which are different from those expressed in the individual from whom the CD8 cells are obtained, regardless of what peptides the Class I molecules contain. This reactivity is the underlying cause of the immune rejection of transplanted organs.

Because it is difficult to find two unrelated persons with exactly the same Class I molecules, some therapeutic approaches take the non-specific approach of "boosting" existing CD8 cells by incubating them *in vitro* with IL-2, a growth factor for T cells. However, this protocol (known as LAK cell therapy or TIL [tumor infiltrating lymphocytes] therapy) will only allow the relatively nonspecific expansion of previously activated CTLs. Most of the IL-2 stimulated cells are irrelevant to combating the disease. In addition, the side effects of LAK cell therapy are often severe. (Greenberg, P., *Adv. in Immunol.* 49:281 (1991); Melief, C., *Adv. Cancer Research* 58:14 (1992); Riddell *et al.*, *Science* 257:238 (1992).

The development of specific CTL-based immunotherapies requires identification of molecules that trigger a CTL response. For example, tumor-associated antigens (TAA). These TAA can be used to elicit immune responses aimed at both

preventing tumor dissemination or recurrence after primary treatment, as well as eradication of already established tumors (Boon, T. *et al.*, *Annual Review of Immunology*, 12:337-365 (1994); Urban, J. L. *et al.*, *Annual Review of Immunology*, 10:617-644 (1994); Rosenberg, S. A., *Immunology Today*, 18:175-182 (1997)). CTL epitopes from various TAA have been identified (Boon, T. *et al.*, *Ann. Rev. Immunol.*, 12:337-365 (1994); Urban, J. L. *et al.*, *Ann. Rev. Immunol.* 10:617-644 (1994); Rosenberg, S. A., *Immunology Today*, 18:175-182 (1997); Fisk, B. *et al.*, *J Exp Med*, 181:2109-2117 (1995); Disis, M. L. *et al.*, *Cancer Research*, 54:1071-1076 (1994); Peoples, G. E. *et al.*, *Proc. Natl. Acad. Sci. USA*, 92:432-436 (1995); Tsang, K. Y. *et al.*, *J. Nat. Cancer Inst.* 87:982-990 (1995); Cox, A. L. *et al.*, *Science*, 264:716-719 (1994); Skipper, J. C. A. *et al.*, *J. Exp. Med.* 183:527-534 (1996)). In most cases, CTLs from cancer patients were used to screen expression gene libraries or peptides eluted from tumor cell MHC molecules. Most of these CTL were from melanoma patients, and the majority of CTL epitopes defined to date are from melanomas. Few epitopes are available to address other more commonly found type of malignancies such as breast, lung, colon and other gastrointestinal carcinomas.

The recent discovery of MHC class I allele specific motifs and the development of peptide-MHC class I binding assays has facilitated the identification of antigenic epitopes that are recognized by CTL. Rammensee, *et al.*, *Immunogenet. 41*:178 (1995); Kubo, *et al.*, J. Immunol. 152:3913 (1994); Ruppert, *et al.*, *Cell 74*:929 (1993); Kast, *et al.*, *J. Immunol. 152*:3904 (1994). Several studies have dissected the cellular and molecular basis of immunodominance in CTL responses in murine systems. Cao, *et al.*, *J. Immunol. 157*:505 (1996); Jameson, *et al.*, *Eur. J. Immunol. 22*:2663 (1992); Wipke, *et al.*, *Eur. J. Immunol. 23*:2005 (1993); Chen, *et al.*, *J. Exp. Med 180*:1471 (1994); Oldstone, M.A., *J. Virol. 65*:6381 (1991); Li, *et al.*, *Eur. J. Immunol. 22*:943 (1992); Sigal, *et al.*, *Molec. Immunol. 32*:623 (1995); Oukka, *et al.*, *J. Immunol 152*:4843 (1994). By contrast, in humans, only dominant epitopes have generally been identified, and definition of subdominant epitopes in humans has been problematic especially for relevant disease settings. *In vitro* immunization studies have in the past generally yielded undramatic results because of the low affinity (avidity) of the CTL elicited and a consequently lack of demonstrable recognition of naturally processed antigen, even in the case of known immunodominant epitopes. Wentworth, *et al.*, *Molec.*

Immunol. 32:603 (1995). Furthermore, deliberate *in vivo* immunization with minimal epitopes has been tried in only very few instances. Vitiello, *et al. J. Clin. Invest. 95*:341 (1995); Marchand, *et al.*, Int. J. Cancer 63:883 (1995); Jaeger, *et al., Int. J. Cancer 66*:162 (1996); Mukherji, *et al., Proc. Natl. Acad. Sci. (USA) 92*:8078 (1995).

In the past, however, no reliable procedures have been available to specifically activate CTLs associated with these diseases. The present invention addresses this and other problems.

SUMMARY OF THE INVENTION

This invention is generally directed to methods of activating cytotoxic T cells (CD8 cells) *in vitro* or *in vivo*. In one embodiment, the method comprises associating desired immunogenic peptides from an antigen with class I MHC molecules on an antigen presenting cells pretreated with a growth factor; and incubating the antigen presenting cells with the cytotoxic T cells in the presence of two or more growth factors, thereby producing activated cytotoxic T cells. A preferred APC is a dendritic cell, especially a dendritic cell that prepared by incubating dendritic cell precursors in the presence of GM-CSF and IL-4. In a preferred embodiment, one growth factor is IL-7 and another is IL-10. IL-7 is preferably added at the start of the incubation step and IL-10 is added one day later.

The invention also provides a method of specifically killing target cells in a human patient, comprising obtaining a fluid sample containing cytotoxic T cells from a patient; contacting the cytotoxic T cells with antigen presenting cells pretreated with one or more pre-treatment growth factors, wherein the antigen presenting cells comprise class I MHC molecules incubating said pretreated antigen presenting cells with the cytotoxic T cells in the presence of desired immunogenic peptides and two or more incubation growth factors, thereby producing activated cytotoxic T cells; contacting the activated cytotoxic T cells with an acceptable carrier, thereby forming a pharmaceutical composition; and administering the pharmaceutical composition to a patient.

The antigen presenting cells having empty MHC class I molecules on their surface are capable of inducing cytotoxic T cells which are useful in the treatment of chronic infectious diseases and cancer. Specifically, this invention provides methods of producing empty MHC class I molecules on antigen presenting cells, loading those empty

MHC class I molecules with selected immunogenic peptides, activating cytotoxic T cells which are specific for killing specific antigen targets. This invention has broad therapeutic application in the treatment of cancers, certain immune diseases and viral diseases. As such the method may further comprise: separating activated CTLs from the antigen presenting cells having the empty MHC class I molecule on its surface; suspending the activated CTLs in an acceptable carrier or excipient as a pharmaceutical composition; and administering the pharmaceutical composition to a patient having the disease.

In preferred embodiments, IL-7, IL-10 and peptide-pulsed dendritic cells (DC) were used to induce primary cytotoxic T lymphocyte (CTL) responses *in vitro*. DC were prepared from peripheral blood mononuclear cells isolated from normal volunteers and pulsed with various HLA-A1 and -A2.1 binding peptides corresponding to known viral and tumor CTL epitopes. These antigen-presenting cells were quite efficient in inducing CTL responses to a hepatitis B virus epitope restricted by the HLA-A2 molecule. Most of the CTLs were not only capable of killing peptide-coated target cells but also endogenously processed antigen, indicating that the CTL possessed both high specificity and affinity to the immunogen. Using peptides corresponding to known tumor associated antigens, CTL induction by DC was significantly enhanced by the addition of recombinant IL-7 and IL-10, but not by IL-12. The peptide-induced CTL were very effective in recognizing and killing tumor cell lines expressing the appropriate tumor antigens, and could be expanded 1000 fold in tissue culture without loss of activity and specificity. The methods for inducing CTL with peptide-coated DC and the combination of cytokines are valuable for both the identification of novel CTL epitopes and for the development of CTL-based adoptive immunotherapies.

The invention also provides methods for defining the entire immunogenic potential of a given antigen in terms of CTL responses, irrespective of whether a given epitope is or is not recognized in the course of a natural disease or infection (that is, whether it is immunodominant or not).

In one embodiment, the gp100 melanoma-associated tumor antigen was selected as a model system to study the diversity of human anti-tumor cytotoxic T cell responses. Peptides corresponding to dominant gp100 HLA-A2.1-restricted CTL epitopes were tested using lymphocytes from normal volunteers and an *in vitro* priming protocol

which utilizes peptide-pulsed dendritic cells as APC, and IL-7 and IL-10 as immune enhancing cytokines. High CTL activity towards both peptide-pulsed target cells and gp100$^+$ melanoma cells was also obtained with 4 out of 5 peptides tested. Thus, the present invention comprises the peptides detected using the methods described herein.

HLA-A2.1-binding peptides from gp100 which do not appear to represent CTL epitopes in melanoma patients, were also tested for their capacity to induce CTL using the *in vitro* priming protocol. Three of six peptides tested induced CTL in lymphocytes from normal volunteers. One of these peptides was also immunogenic for lymphocytes derived from a melanoma patient in remission. Because these three CTL epitopes were not recognized in the natural immune response in melanoma patients but do appear as immunogens when peptides are used to induce the T-cell response, they may be considered as typical "subdominant" epitopes.

The present invention provides an alternative approach to tumor epitope identification which does not require the use of patient's CTL, but relies instead on the identification of MHC-binding peptides from known TAA and on novel *in vitro* priming protocols. Specifically, peptides selected on the basis of MHC binding motifs and on their binding capacity to MHC molecules, are tested for their ability to elicit tumor-reactive CTL *in vitro* using lymphocyte cultures from normal individuals. Following this "reverse immunology" approach, several CTL epitopes from TAA expressed in melanoma tumors have been identified. In the examples, this methodology is applied to identify CTL epitopes derived from various TAA expressed on solid adenocarcinomas (Weynants, P. *et al.*, *International Journal of Cancer* 56:826-829 (1994); De Plaen, E. *et al.*, *Immunogenetics* 40: 360-369 (1994); Vincent, R. G. *et al.*, *Cardiovasc. Surg.* 66: 320-328 (1978); Shavely, J. *et al.*, CRC Critical Reviews in Oncology and Hematology 2:355-399 (1985); Thompson, J., *et al.*, *Journal of Clinical and Laboratory Analytics* 5:344-366 (1991); Sikorska, H. *et al.*, *Cancer Detection and Prevention* 12:321-355 (1988); Muraro, R. *et al.*, *Cancer Research* 45: 5769-5780 (1985); Steward, A. M. *et al.*, *Cancer* 33:1246-1252 (1974); Slamon, D. J. *et al.*, *Science* 244:707-712 (1989); Yokota, J. *et al.*, *Lancet* 1:765-767 (1986)) such as, *MAGE2*, *MAGE3*, carcinoembryonic antigen (CEA), and HER2/*neu*. These results are relevant for the development of epitope-based immunotherapy of high incidence tumors such as breast, lung, colon and gastric carcinomas.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. **Antigen specificity and CTL responses to a *MAGE-3* epitope in lymphocyte cultures after expansion *in vitro*.**

One positive CTL culture (from experiment described in Table III) was expanded with either anti-CD3 monoclonal antibodies (A) or *MAGE-3*$_{161}$ peptide (B) as described in Materials and Methods. On day 13 the cytotoxic activity was tested against the following targets: ■, Steinlin plus peptide; □, Steinlin minus peptide; ●, 938mel (A1+, *MAGE-3*$^+$); ○, peptide, .888mel (A1+, *MAGE-3*$^-$). On day 13, the cells stimulated with anti-CD3 antibodies (A)expanded 1300 fold and the cells expanded with peptide (B)expanded 820 fold.

Figure 2. **Antigen specificity and CTL response to gp100 peptide G9$_{280}$.**

CD8$^+$ lymphocytes from a normal volunteer were stimulated *in vitro* with G9$_{280}$-pulsed autologous DC. A: After a CTL line was established by weekly re-stimulation with peptide-pulsed monocytes, the lytic activity was measured using various target cell lines: ■, .221 (A2.1) - no peptide; □, .221(A2.1) plus G9$_{280}$; ○, 624mel (A2$^+$, gp100$^+$); ▲, A375mel (A2$^+$, gp100$^-$); ◇, 697mel (A2$^+$, gp100$^+$); △, 677mel (A2$^+$, gp100$^+$). B: Antigen specificity demonstrated by cold target inhibition studies. Lysis of 624mel (A2$^+$, gp100$^+$) at an E:T ratio of 30:1 by G9$_{280}$-specific CTL line was blocked by the following cold (unlabeled) target cells: □, .221 (A2.1) plus G9$_{280}$; ■, .221 (A2.1) plus control HLA-A2.1-binding peptide (FLPSDFFPSV); ○, .221(A2.1) - no peptide.

Figure 3. **Antigen specificity and CTL res ponses to gp100 peptides G9$_{178}$ (A)and G10$_{177}$ (B).**

CD8+ lymphocytes from a normal volunteer were stimulated *in vitro* with gp100 peptides loaded onto autologous DC. CTL lines were produced and tested with various "Cr labeled target cell lines in the presence and absence of unlabelled cold targets (Inhibitors) at an inhibitor:target ratio of 20: 1. Experimental conditions for both panels: □, .221(A2.1) plus gp100 peptide (G9$_{178}$ in A and G10$_{177}$ in B); ○, .221(A2.1) - no peptide; ▲, 624mel (A2+, gp100+); Δ, A375mel (A2+, gp100); ◇, 624mel plus .221(A2.1)

inhibitors pulsed with gp100 peptide ($G9_{178}$ in A and $G10_{177}$ in B); ∇, 624 mel plus .221(A2.1) inhibitors pulsed with control peptide (FLPSDFFPSV).

Figure 4. **CTL lines specific for gp100 peptides $G9_{178}$ and $G10_{177}$, recognize two separately different epitopes.**

CTLs elicited with peptide $G9_{178}$, (A) or $G10_{177}$ (B) were tested for their capacity to kill these targets: □, .221(A2.1) plus $G10_{177}$; ○, .221 (A2. 1) plus $G9_{178}$; Δ, .221 (A2. 1) - no peptide; ▲, 624mel (A2+, gp100+); ◇, A375mel (A2+, gp100).

Figure 5. **CTL response to gp100 peptide $G10_{570}$.**

CTL were elicited using CD8+ lymphocytes from a normal volunteer using peptide-pulsed autologous DC. A: The $G10_{570}$ -specific CTL line was tested for its ability to kill the following targets: □, .221(A2.1) plus $G10_{570}$; ○, .221(A2.1) - no peptide; ■, 697mel (A2+, gp100+); ∇, 624mel (A2+, gp100+); •, A375mel (A2+, gp100); ▲, 677mel (A2+, gp100+). *B:* Cold target inhibition of the lysis of 624mel (A2+, gp100+) cells. Killing of ^{51} Cr labeled 624mel cells was blocked by cold (unlabeled) .221 (A2.1) cells pulsed with $G10_{570}$ (▾) or control peptide (∇ -, FLPSDFFPSV) at an inhibitor:target ratio of 20:1.

Figure 6. **Induction of melanoma-reactive CTL with patient's lymphocytes using a peptide corresponding to a subdominant epitope.**

Blood lymphocytes from a melanoma patient were stimulated *in vitro* with autologous DC pulsed with peptide $G10_{177}$, and after 3 cycles of antigen-restimulation, the T-cell line was assayed for its lytic activity against these targets: □,.221(A2.1) plus $G10_{177}$; ■,.221(A2.1) - no peptide; ○, 624mel (A2+, gp100+); ▲, A375mel (A2+, gp100). Antigen specificity was further demonstrated by cold target inhibition of the lysis of 624mel cells by the addition of a 20:1 excess of unlabeled .221(A2.1) pulsed with $G10_{177}$, (◇); or unlabeled .221 (A2.1) pulsed with an HLA-A2.1-binding control peptide (Δ, FLPSDFFPSV).

Figure 7. **Recognition of various tumor types by a *MAGE2* specific CTL clone.**

The *MAGE2*[10_{157}] specific CTL clone was tested for its lytic activity against the following target cells: O, .221A2.1 pulsed with *MAGE2*[10_{157}]; •,.221A2.1 without peptide; Δ, 624mel (melanoma, A2$^+$, *MAGE2*$^+$); ❑, KATO-III (gastric Ca, A2$^+$, *MAGE2*$^+$); ◇, SW403 (colon Ca, A2$^+$, *MAGE2*?); ■, WiDr (colon Ca, A2, *MAGE2*?); ▲, 888mel (melanoma, A2, *MAGE2*$^-$).

Figure 8. **Cold target inhibition assays to demonstrate the antigen specificity of *MAGE2*[10_{157}] specific CTL clone.**

The tumor cell lines 624mel (*A*), SW403 (*B*) or KATO-III (*C*) were ^{51}Cr labeled and mixed at various Inhibitors / Target ratios with following cold targets: O, .221A2.1 pulsed with *MAGE2*[10_{157}]; ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide (HBc_{18-27}); •, .221A2.1 without peptide. The effectors/target ratio used were 8:1 (A), 4:1 (B) or 5:1 (C).

Figure 9. **Recognition of various tumor types by a *MAGE3* specific CTL clone.**

MAGE3[9_{121}] specific CTL clone was tested for cytotoxicity using following targets: O, .221A2.1 pulsed with MAGE3[9_{112}]; •,.221A2.1 without peptide; Δ, 624mel (melanoma, A2$^+$, MAGE3$^+$); □, KATO-III (gastric Ca, A2$^+$, *MAGE3*$^+$); ■, SW403 (colon Ca, A2$^+$, MAGE3$^+$); ■, WiDr (colon Ca, A2$^-$, *MAGE3*+); ▲, 888mel (melanoma, A2$^-$, *MAGE3*$^-$).

Figure 10. **Cold target inhibition assays to demonstrate the antigen specificity of a MAGE3[9_{112}]-specific CTL clone.**

The tumor cell lines 624mel (*A*), SW403 (*B*) or KATO-III (*C*) were ^{51}Cr labeled and mixed at various Inhibitors / Target ratios with following cold targets: O, .221 A2.1 pulsed with MAGE3[9_{112}]; ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide (HBc_{18-27}); •, .221A2.1 without peptide. The effectors/target ratio used were 8:1 (A), 8:1 (B) or 5:1 (C).

Figure 11. **Recognition of various tumor types by a CEA specific CTL clone.**

The CEA[9_{691}] specific CTL clone was tested for its lytic activity against following target cell lines: O, .221A2.1 pulsed with CEA[9_{691}]; ●, .221A2.1 without peptide; Δ, KATO-III (gastric Ca, $A2^+$, CEA^+); ◇, SW403 (colon Ca, $A2^+$, CEA^+); ▲, HT-29 (colon Ca, $A2^-$, CEA^+).

Figure 12. **Cold target inhibition assays to demonstrate the antigen specificity of CEA specific CTL.**

CEA[9_{691}] specific CTL were tested at the effectors/target ratio of 15:1 in both experiments. The tumor cell lines KATO-III (*A*) or SW403 (*B*) were ^{51}Cr labeled and mixed at different Inhibitors / Target ratios with following cold targets: 0, .221A2.1 pulsed with CEA[$9_{,691}{}^+$]; ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide ($HBc_{18\text{-}27}$).

Figure 13. **HER2[9_{435}] specific CTL can kill tumor cells.**

(A): HER2[9_{435}] specific CTLwere used as effector cells to test for the lysis of following target cell lines. O, .221A2.1 pulsed with HER2[9_{435}]; ●, .221A2.1 without peptide; Δ, SW403 (colon Ca, $A2^+$, HER-2/*neu*$^+$); ▲, HT-29 (colon Ca, $A2^-$, HER-2/*neu*$^+$). **(B)**: Antigen specificity demonstrated by cold target inhibition assay. Lysis of ^{51}Cr labeled SW403 cells at an effectors / target ratio of 10:1 by the HER2[9_{435}] specific CTL was blocked at various Inhibitors / Target ratios by the following cold targets: (D, .221A2.1 pulsed with HER2[9_{435}]; ▲. 221A2.1 pulsed with irrelevant A2.1 binding peptide ($HBC_{18\text{-}27}$); ●. .221A2.1 without peptide.

Figure 14. **Demonstration of tumor reactivity of HER2[9_5] specific CTL.**

(A): The HER2[9_5]-specific CTL line was used as effector to test the lysis of following target cell lines. O,. .221A2.1 pulsed with HER2[9_5]; ●, .221A2.1 without peptide; Δ, SW403 (colon Ca, $A2^+$, HER-2/*neu*$^+$); ▲, HT-29 (colon Ca, $A2^-$, HER-2/*neu*$^+$).
(B): Antigen specificity demonstrated by a cold target inhibition assay. Lysis of ^{51}Cr-labeled SW403 at an effector/target ratio of 10:1 by the HER2[9_5]-specific CTL line was blocked at various Inhibitors / Target ratios by the following cold targets: O, .221A2.1 pulsed with HER2[9_5]; ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide ($HBc_{18\text{-}27}$); ●, .221A2.1 without peptide.

Figure 15. **Peptide analogs can induce tumor-reactive CTL.**
(A): The CEA[9_{24}]M2V9 specific CTL clone was tested for its cytolytic activity against following target cell lines: O, .221A2.1 pulsed with CEA[9_{24}]M2V9;. ●, .221A2.1 without peptide; Δ, SW403 (colon Ca, $A2^+$, CEA_+); ■, KATO-III (gastric Ca, $A2^+$, CEA^+); ▲, HT-29 (colon Ca, $A2^-$, CEA^+).
(B): Antigen specificity demonstrated by cold target inhibition assay. Lysis of ^{51}Cr-labeled SW403 cells at an effector/target ratio of 2:1 by the CEA[9_{24}]M2V9 specific CTL clone was blocked at various Inhibitors / Target ratios by the following cold targets: O, .221A2.1 pulsed with CEA[9_{24}]M2V9; ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide (HBc_{18-27}); ●, .221A2.1 without peptide.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "peptide" is used interchangeably with "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of adjacent amino acids.

An "immunogenic peptide" is a peptide which comprises an allele-specific motif such that the peptide will bind the MHC allele and be capable of inducing a CTL response. Thus, immunogenic peptides are capable of binding to an appropriate class I MHC molecule and inducing a cytotoxic T cell response against the antigen from which the immunogenic peptide is derived.

The term "residue" refers to an amino acid or amino acid mimetic incorporated in an oligopeptide by an amide bond or amide bond mimetic.

The term "pretreatment growth factors" refers to growth factors added to antigen presenting cells prior to incubating the antigen presenting cells with CTLS, peptides and growth factors that are used to expand the CTLs. The term "incubation growth factors" refers to growth factors that are added as APCs and CTLs are incubated together to expand specific CTLS.

Our invention further comprises a general strategy to define the full immunological potential of a given antigen. As a result, and in conjunction with data derived from clinical samples, the dominance and subdominance of CTL epitopes

associated to pathological states such as chronic viral infections and cancer can now be defined. We anticipate that experimental strategies similar to the one detailed herein could help to shed some light on the mechanisms involved in the pathogenesis of infection, cancer, and autoimmune diseases.

The present invention provides a new *in vitro* primary immunization assay and its application to identify human CTL epitopes derived from tumor-associated antigens. In one embodiment, the invention entails stimulation of human PBMC with peptide-pulsed dendritic cells in the presence of IL-7 and IL-10. Utilizing this protocol we have defined 3 novel subdominant HLA-A2.1-restricted CTL epitopes derived from the gp100 melanoma associated tumor antigen ($G9_{178}$, $G10_{177}$ and $G10_{570}$). Since only 6 of 12 potential subdominant epitopes have been tested to date following our dendritic cell priming *in vitro* immunization protocol, it is likely that additional gp100 derived epitopes can also be identified. The experimental protocol described herein allows one to define the CTL epitopes contained within a given tumor antigen (regardless of whether they re immunodominant or not) and fully exploit the immunological potential of the tumor antigen itself.

The present invention also provides methods for ascertaining the role of immunodominance in the specific area of anti-tumor CTL responses. It has been pointed out that some of the CTL epitopes recognized by cancer patients appear to bind their MHC class I restriction element with relatively weaker affinities than epitopes recognized, for example, in acute viral infections (Kawakami, *et al., J. Immunol. 154*:3961 (1995); Celis, *et al. Sem. Cancer Biol. 6*:329 (1995)). These results suggested the possibility that some of the CTL reactivities directed against the highest MHC class I binding epitopes might have been inactivated by thymic education or peripheral tolerance and dominant tumor epitopes might be in fact relatively low affinity binders. Indeed, in a recent survey, we found peptides recognized by cancer patients bound their corresponding MHC molecules with an average affinity of 170 $\pm$ 52 nM (n=12), somewhat lower than the epitopes recognized in a viral disease setting (average affinity of 49 $\pm$ 15 nM (n= 15). Epitopes binding in the 11 to 500 nM range were found in both dominant and subdominant groups (Tables II and IV), and the 2 highest affinity binding peptides $G9_{154}$, and $G10_{476}$ (Table I) correspond to immunodominant CTL epitopes recognized by melanoma patient's TIL. Furthermore, the dominant gp100 epitopes elicited

anti-melanoma CTL responses in 10-38% of all lymphocyte cultures, while the subdominant epitopes induced CTL that could kill melanoma cells less frequently (2-3% of the cultures).

In vivo and *in vitro* CTL inductions using both dominant and subdominant epitopes yields a more vigorous and diverse immune response. Polyvalent vaccines containing mixtures of epitopes from several TAA may overcome the need for complex mixtures of whole proteins or genes. Immunization with subdominant epitopes could also be used to focus CTL responses toward specific conserved sequences and thus prevent the emergence of tumor variants during the course of disease.

The present invention also provides that it is possible to induce tumor-reactive CTL from blood of cancer patients specific for both dominant and subdominant epitopes. These CTL could be expanded in culture to approximately 1 X 10^9 cells (data not shown), still retained antigen specificity and could thus be used for adoptive therapies. Immune therapy using peptide-primed CTL from blood may be a convenient (since no tumor biopsy is required), and reliable (because of the high degree of specificity) alternative to the already promising current TIL based immunotherapies (Rosenberg, *et al.*, *J. Natl. Cancer Inst. 86*:1159 (1994)).

The procedures of the present invention depend in part upon the determination of epitopes recognized by CTLs capable of eliminating target infected cells. One approach to identification of these epitopes is the identification of allele-specific peptide motifs associated with a particular disease for human Class I MHC allele subtypes. The MHC class I antigens are encoded by the HLA-A, B, and C loci. HLA-A and B antigens are expressed at the cell surface at approximately equal densities, whereas the expression of HLA-C is significantly lower (perhaps as much as 10-fold lower). Each of these loci have a number of alleles. A large number of cells with defined MHC molecules, particularly MHC Class I molecules, are known and readily available. These cells can be used to identify particular allele specific motifs associated with target diseases.

The allele-specific motifs are then used to define T cell epitopes from any desired antigen, particularly those associated with human viral diseases or cancers, for which the amino acid sequence of the potential antigen targets is known. This general approach is described in detail in copending and commonly assigned applications

U.S.S.N. 07/926,666 and U.S.S.N. 08/027,146, which are incorporated herein by reference.

Potential epitopes on a number of target proteins can be identified in this manner. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B core, surface and polymerase antigens (HBVc, HBVs, HBVp), hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (*e.g.*, MAGE-1), human immunodeficiency virus (HIV) antigens, human papilloma virus (HPV) antigens, cytomegalovirus (CMV), herpes simplex virus (HSV), and other oncogene products (c-Erb B_2, CEA, p 53-breast/ovary).

These approaches typically involve isolation of peptides from a particular MHC molecule and sequencing the peptides to determine the relevant motif. Buus *et al.*, *Science*, 242:1065 (1988) first described a method for acid elution of bound peptides from MHC. Subsequently, Rammensee and his coworkers (Falk *et al.*, *Nature*, 351:290 (1991) developed an approach to characterize naturally processed peptides bound to class I molecules. Other investigators have successfully achieved direct amino acid sequencing of the more abundant peptides in various HPLC fractions by conventional automated sequencing of peptides eluted from B type class I molecules (Jardetzky, *et al.*, *Nature*, 353:326 (1991) and of the A2.1 type by mass spectrometry (Hunt, *et al.*, *Science*, 225:1261 (1992). A review of the characterization of naturally processed peptides found on MHC Class I molecules is presented by Rötzschke and Falk (Rötzschke and Falk, *Immunol. Today*, 12:447 (1991).

Definition of motifs specific for different class I alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino acid sequence is known. Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs. The epitopic sequences are then synthesized. The capacity to bind MHC Class molecules is measured in a variety of different ways using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorimetry, peptide-dependent class I assembly assays, and inhibition of CTL recognition by peptide competition. Other alternatives described in the literature include inhibition of antigen presentation (Sette, *et al.*, *J. Immunol.*, 141:3893 (1991), in vitro assembly assays

(Townsend, *et al.*, *Cell*, 62:285 (1990)), and FACS based assays using mutated ells, such as RMA.S (Melief, *et al.*, *Eur. J. Immunol.*, 21:2963 [1991]).

Next, peptides that test positive in the MHC class I binding assay are assayed for the ability of the peptides to induce specific primary or secondary CTL responses *in vitro*. For instance, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. For secondary responses, antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, *et al.*, *J. Exp. Med.*, 166:182 (1987); Boog, *Eur. J. Immunol.*, 18:219 [1988]).

Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides, such as the mouse cell lines RMA-S (Kärre, *et al.*. *Nature*, 319:675 (1986); Ljunggren, *et al.*, *Eur. J. Immunol.*, 21:2963-2970 (1991)), and the human somatic T cell hybridoma, T-2 (Cerundolo, *et al.*, *Nature*, 345:449-452 (1990)) and which have been transfected with the appropriate human class I genes are conveniently used, when peptide is added to them, to test for the capacity of the peptide to induce *in vitro* primary CTL responses. These empty MHC cells are preferable for inducing a primary response since the density of MHC-peptide complexes on the surface of the antigen presenting cell will be greater. Other eukaryotic cell lines which could be used include various insect cell lines such as mosquito larvae (ATCC cell lines CCL 125, 126, 1660, 1591, 6585, 6586), silkworm (ATTC CRL 8851), armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila cell lines such as a Schneider cell line that have been transfected with the appropriate human class I MHC allele encoding genes and the human B_2 microglobulin genes.

Once the appropriate epitope is determined, immunogenic peptides comprising the motif required for MHC binding and the epitope recognized by the CTL are synthesized. The immunogenic peptides can be prepared synthetically, or by recombinant DNA technology or isolated from natural sources such as whole viruses or tumors. One of skill will recognize that the immunogenic peptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be desirable to optimize peptides of the invention to a length of 9 or 10 amino acid residues, commensurate in size with endogenously processed viral peptides or tumor cell peptides that are bound to MHC class I molecules on the cell surface.

Peptides having the desired activity may be modified as necessary to provide certain desired attributes, *e.g.*, improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance, the peptides may be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, *e.g.*, one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. The effect of single amino acid substitutions may also be probed using D-amino acids. Such modifications may be made using well known peptide synthesis procedures.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, *Solid Phase Peptide Synthesis*, 2d. ed., Pierce Chemical Co. (1984), *supra*.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook *et al.*, *Molecular Cloning*, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982), which is incorporated herein by reference. Fusion proteins which comprise one or more peptide sequences of the invention can also be used to present the appropriate T cell epitope.

The immunogenic peptides are then used to activate CTL *ex vivo*. The *ex vivo* therapy methods of the present invention and pharmaceutical compositions thereof are useful for treatment of mammals, particularly humans, to treat and/or prevent viral infection, immune disorders and cancer. Examples of diseases which can be treated using the *ex vivo* therapy methods of the invention include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma, lymphoma, CMV, condyloma acuminatum, breast and ovarian cancer, colon, lung cancer and HSV.

For therapeutic use, therapy should begin at the first sign of viral infection or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting levels of CTL at least until symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by boosting doses may be required.

Treatment of an infected individual with the methods of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the methods are useful for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, the compositions can be targeted to them, minimizing the need for administration to a larger population.

The methods of the present invention can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers.

Ex vivo CTL responses to a particular pathogen (infectious agent or tumor antigen) are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) loaded with the appropriate immunogenic peptide. After an appropriate incubation time (typically 3-12 weeks) in which the CTLp are activated and mature and expand into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell). Infusion of the cells into the patient may include a T cell growth factor such as interleukin 2 (IL-2). In order to optimize the *in vitro* conditions for the generation of specific cytotoxic T cells, the culture of stimulator cells is maintained in an appropriate serum-free medium which may include one or more growth factors such as IL-2, IL-4, IL-7 and IL-12.

Peripheral blood lymphocytes are conveniently isolated following simple venipuncture or leukapheresis of normal donors or patients and used as the responder cell sources of CTLp. In one embodiment particularly for secondary CTL responses, the appropriate APC are incubated with 10-100 μM of peptide in serum-free media for 4 hours under appropriate culture conditions. The peptide-loaded APC are then incubated with the responder cell populations *in vitro* for 7 to 10 days under optimized culture conditions. For primary CTL induction, APC expressing empty MHC would be used to stimulate naive CTLp. In this case the CTL would be stimulated more frequently (1-2 times).

Positive CTL activation can be determined by assaying the cultures for the presence of CTLs that kill radiolabeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed form of the relevant virus or tumor antigen from which the peptide sequence was derived.

Specificity and MHC restriction of the CTL of a patient can be determined by a number of methods known in the art. For instance, CTL restriction can be determined by testing against different peptide loaded target cells expressing human MHC class I alleles shared with the HLA phenotype of the donor CTL. The peptides that test positive in the MHC binding assays and give rise to specific CTL responses are identified as immunogenic peptides.

As mentioned above, the induction of CTL *in vitro* requires the specific recognition of peptides that are bound to allele specific MHC class I molecules on APC. The number of specific MHC/peptide complexes per APC determines the level of stimulation of CTL, particularly during the primary immune response. While small amounts of peptide/MHC complexes per cell are sufficient to render a cell susceptible to lysis by CTL, or to stimulate a secondary CTL response, the successful activation of a CTLp during primary response requires a significantly higher number of MHC/peptide complexes.

Since mutant cell lines capable of expressing empty MHC do not exist for every human MHC allele, it is advantageous to use a technique to remove endogenous MHC-associated peptides from the surface of APC, followed by loading the resulting empty MHC molecules with the immunogenic peptides of interest. The use of non-transformed (non-tumorigenic), non-infected cells, and preferably, autologous cells of

patients as APC is desirable for the design of CTL induction protocols directed towards development of *ex vivo* CTL therapies. This present invention provides novel methods generating empty class I MHC which can then be loaded with an appropriate immunogenic peptide by stripping the endogenous MHC-associated peptides from the surface of APC or through cold temperature incubation (37°C→26°C)followed by the loading of desired peptides.

A stable MHC class I molecule is a trimeric complex formed of the following elements: 1) a peptide usually of 8 - 10 residues, 2) a transmembrane heavy polymorphic protein chain which bears the peptide-binding site in its α1 and α2 domains, and 3) a non-covalently associated non-polymorphic light chain, β_2 microglobulin. Removing the bound peptides and/or dissociating the β_2 microglobulin from the complex renders the MHC class I molecules nonfunctional and unstable, resulting in rapid degradation at 37°C. Almost all MHC class I molecules isolated from PBMCs have endogenous peptides bound to them. Therefore, the first step to prepare APC for primary CTL induction is to remove all endogenous peptides bound to MHC class I molecules on the APC without causing degradation or cell death before exogenous peptides can be added.

Two possible ways to generate free MHC class I molecules include lowering the culture temperature from 37°C to 26°C overnight to allow MHC class I without peptides to be expressed and stripping the endogenous peptides from the cell using a mild acid treatment. The mild acid treatment releases previously bound peptides into the extracellular environment allowing new exogenous peptides to bind to the empty class I molecules. The overnight cold-temperature incubation at 26°C which may slow the cell's metabolic rate enables expression of stable empty class I molecules which then bind exogenous peptides efficiently. It is also likely that cells not actively synthesizing MHC molecules (*e.g.*, resting PBMC) would not produce high amounts of empty surface MHC molecules by the cold temperature procedure.

Extraction of the peptides is accomplished by harsh acid stripping using trifluoroacetic acid, pH 2, or acid denaturation of the immunoaffinity purified class I-peptide complexes. These methods are not feasible for CTL induction, since it is important to remove the endogenous peptides while preserving APC viability and an optimal metabolic state which is critical for antigen presentation. Mild acid solutions of

pH 3 such as glycine or citrate-phosphate buffers have been used to identify endogenous peptides and to identify tumor associated T cell epitopes. The treatment is especially effective, in that only the MHC class I molecules are destabilized (and associated peptides released), while other surface antigens remain intact, including MHC class II molecules. (Suguwara, S., *et al.*, *J. Immunol. Meth.* 100:83 (1987)). Most importantly, treatment of cells with the mild acid solutions do not affect the cell's viability or metabolic state. The mild acid treatment is rapid since the stripping of the endogenous peptides occurs in two minutes at 4°C and the APC is functional after the appropriate peptides are loaded. The technique is utilized herein to make peptide-specific APCs for the generation of primary antigen-specific CTL. The resulting APCs are efficient in inducing peptide-specific CTL.

Typically in a primary response prior to incubation of the APCs with the CTLp to be activated, an amount of antigenic peptide is added to the APCs or stimulator cell culture, of sufficient quantity to become loaded onto the human Class I molecules to be expressed on the surface of the APCs. In the present invention, a sufficient amount of peptide is an amount that will allow about 200 or more human Class I MHC molecules loaded with peptide to be expressed on the surface of each stimulator cell. Preferably, the stimulator cells are incubated with 5-100μg/ml peptide.

Resting or precursor CTLs are then incubated in culture with the appropriate APCs for a time period sufficient to activate the CTLs. The CTLs are activated in an antigen-specific manner. The ratio of precursor CTLs to APCs may vary from individual to individual and may further depend upon variables such as the amenability of an individual's lymphocytes to culturing conditions and the nature and severity of the disease condition or other condition for which the within-described treatment modality is used. Preferably, however, the CTL:APC (i.e. responder to stimulator) ratio is in the range of about 10:1 to 100:1. The CTL/APC culture may be maintained for as long a time as is necessary to stimulate a therapeutically useable or effective number of CTL.

Activated CTL may be effectively separated from the APC using one of a variety of known methods. For example, monoclonal antibodies specific for the APCs, for the peptides loaded onto the stimulator cells, or for the CTL (or a segment thereof) may be utilized to bind their appropriate complementary ligand. Antibody-tagged cells

may then be extracted from the admixture via appropriate means, *e.g.*, via well-known immunoprecipitation or immunoassay methods.

Effective, cytotoxic amounts of the activated CTLs can vary between *in vitro* and *in vivo* uses, as well as with the amount and type of cells that are the ultimate target of these killer cells. The amount will also vary depending on the condition of the patient and should be determined via consideration of all appropriate factors by the practitioner. Preferably, however, about 1 X 10^6 to about 1 X 10^{12}, more preferably about 1 X 10^8 to about 1 X 10^{11}, and even more preferably, about 1 X 10^9 to about 1 X 10^{10} activated CTLS are utilized for adult humans, compared to about 5 X 10^6 - 5 X 10^7 cells used in mice.

As discussed above, the activated CTLS may be harvested from the cell culture prior to administration of the cells to the individual being treated. It is important to note, however, that unlike other present treatment modalities, the present method uses a cell culture system that does not contain transformed or tumor cells. Therefore, if complete separation of antigen-presenting cells and activated CTLS is not achieved, there is no inherent danger known to be associated with the administration of a small number of stimulator cells, whereas administration of mammalian tumor-promoting cells may be extremely hazardous.

One embodiment of the present invention uses the APC generated by the *in vitro* techniques of this application for therapy *against* CTL *in vivo*. In this embodiment, the APC are a patient's cells (*e.g.*, the peripheral blood cells) which are stripped of their natural antigenic peptides and loaded with a peptide of choice which is conjugated to a toxin (*e.g.* ricin A chain or pseudomonas toxin). The APCs are then re-introduced into the patient, where they will be bound by the endogenous CTLs that are specific for the antigenic peptide. The coupled toxin will kill the activated CTL that are harmful i.e. those which stimulate transplant rejection after it binds the APC. Such directed CTL killing is broadly useful for treating tissue-transplantation rejection and auto-immune disorders, which are mediated through CTL. The treatment regime will vary depending upon the specific disorder to be treated and the judgement of the treating physician.

Methods of re-introducing cellular components are known in the art and include procedures such as those exemplified in U.S. Patent No. 4,844,893 to Honsik, *et al.* and U.S. Patent No. 4,690,915 to Rosenberg, which are incorporated herein by

reference. For example, administration of activated CTLs via intravenous infusion is appropriate.

The identification of those peptide sequences which provide an immunogenic potential for CTL precursors (CTLp) specific for viral and neoplastic diseases would facilitate the development of novel epitope defined immunotherapies, such as peptide-based vaccines and antigen-specific adoptive cell therapies.

The pan DR-binding peptides of the present invention and pharmaceutical and vaccine compositions thereof can be administered to mammals, particularly humans, for prophylactic and/or therapeutic purposes. The pan DR peptides can be used to enhance immune responses against other immunogens administered with the peptides. For instance, CTL/pan DR mixtures may be used to treat and/or prevent viral infection and cancer. Alternatively, immunogens which induce antibody responses can be used. Examples of diseases which can be treated using the immunogenic mixtures of pan DR peptides and other immunogens include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma, lymphoma, CMV and condyloma acuminatum.

The pan DR-binding peptides may also be used to treat a variety of conditions involving unwanted T cell reactivity. Examples of diseases which can be treated using pan DR-binding peptides include autoimmune diseases (*e.g.*, rheumatoid arthritis, multiple sclerosis, and myasthenia gravis), allograft rejection, allergies (*e.g.*, pollen allergies), lyme disease, hepatitis, LCMV, post-streptococcal endocarditis, or glomerulonephritis, and food hypersensitivities.

In therapeutic applications, the immunogenic compositions or the pan DR-binding peptides of the invention are administered to an individual already suffering from cancer, autoimmune disease, or infected with the virus of interest. Those in the incubation phase or the acute phase of the disease may be treated with the pan DR-binding peptides or immunogenic conjugates separately or in conjunction with other treatments, as appropriate.

In therapeutic applications, compositions comprising immunogenic compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and to cure or at least partially arrest symptoms and/or complications. Similarly, compositions comprising pan DR-binding peptides are administered in an amount sufficient to cure or at least partially arrest the

symptoms of the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, *e.g.*, the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

Therapeutically effective amounts of the immunogenic compositions of the present invention generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 1.0 μg to about 10,000 μg of peptide for a 70 kg patient, usually from about 100 to about 8000 μg, and preferably between about 200 and about 6000 μg. These doses are followed by boosting dosages of from about 1.0 μg to about 1000 μg of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood.

It must be kept in mind that the compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the conjugates, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these compositions.

For prophylactic use, administration should be given to risk groups. For example, protection against malaria, hepatitis, or AIDS may be accomplished by prophylactically administering compositions of the invention, thereby increasing immune capacity. Therapeutic administration may begin at the first sign of disease or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by boosting doses may be required.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection,

for instance, as described herein, the composition can be targeted to them, minimizing need for administration to a larger population.

The peptide mixtures or conjugates can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers. It is important to provide an amount of immuno-potentiating peptide in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic infection, a representative dose is in the range of about 1.0 μg to about 5000 μg, preferably about 5 μg to 1000 μg for a 70 kg patient per dose. Immunizing doses followed by boosting doses at established intervals, *e.g.*, from one to four weeks, may be required, possibly for a prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

The pharmaceutical compositions for therapeutic or prophylactic treatment are intended for parenteral, topical, oral or local administration. Typically, the pharmaceutical compositions are administered parenterally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Because of the ease of administration, the vaccine compositions of the invention are particularly suitable for oral administration. Thus, the invention provides compositions for parenteral administration which comprise a solution of the peptides or conjugates dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.9% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.*

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at

or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected.

The peptides and conjugates of the invention may also be administered via liposomes, which serve to target the conjugates to a particular tissue, such as lymphoid tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, *e.g.*, a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired peptide or conjugate of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, *e.g.*, liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9, 467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

For targeting to the immune cells, a ligand to be incorporated into the liposome can include, *e.g.*, antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide or conjugate may be administered intravenously, locally, topically, *etc.* in a dose which varies according to, inter alia, the manner of administration, the conjugate being delivered, and the stage of the disease being treated.

Alternatively, DNA or RNA encoding one or more PADRE peptides and a polypeptide containing one or more CTL epitopes or antibody inducing epitopes may be introduced into patients to obtain an immune response to the polypeptides which the nucleic acid encodes. Wolff *et. al.*, *Science* 247: 1465-1468 (1990) describes the use of nucleic acids to produce expression of the genes which the nucleic acids encode.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more conjugates of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of conjugates are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, *e.g.*, lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of an immunogenic pan DR peptide or a CTL\pan DR peptide conjugate as described herein. The conjugate(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the virus or tumor cells. Useful carriers are well known in the art, and include, *e.g.*, thyroglobulin, albumins such as bovine serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are

materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P_3CSS. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

Vaccine compositions containing the pan DR peptides of the invention are administered to a patient susceptible to or otherwise at risk of disease, such as viral infection or cancer to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, *etc.*, but generally range from about 1.0 μg to about 5000 μg per 70 kilogram patient, more commonly from about 10 μg to about 500 μg per 70 kg of body weight.

In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to the virus of interest, particularly to viral envelope antigens. For instance, PADRE peptides can be combined with hepatitis vaccines to increase potency or broaden population coverage. Suitable hepatitis vaccines that can be used in this manner include, Recombivax HB ® (Merck) and Engerix-B (Smith-Kline).

For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL response. Vaccinia vectors and methods useful in immunization protocols are described in, *e.g.*,U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover *et al.*, *Nature* 351, 456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, *e.g.*,

Salmonella typhi vectors and the like, will be apparent to those skilled in the art from the description herein.

Antigenic conjugates may be used to elicit CTL ex vivo, as well. The resulting CTL, can be used to treat chronic infections (viral or bacterial) or tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a peptide vaccine approach of therapy. Ex vivo CTL responses to a particular pathogen (infectious agent or tumor antigen) are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate immunogenic peptide. After an appropriate incubation time (typically 1-4 weeks), in which the CTLp are activated and mature and expand into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell).

We have previously described a procedure to screen various class I MHC-binding peptides for their ability to elicit CTL responses *in vitro* using peripheral blood mononuclear cells (PBMC) from normal volunteers (Celis, *et al.*, *Proc. Natl. Acad. Sci. (USA) 91*:2105 (1994)). The process utilized CD8 purified lymphocytes as a source of CTLp and peptide-pulsed antigen presenting cells (APC) that were prepared by activating PBMC with *Staphyloccocus aureus* Cowan-I (SAC-1). This protocol allowed us and other groups to identify several new tumor-specific CTL epitopes. In an experimental model system using an HLA-A2.1-binding peptide from hepatitis B virus (HBV) we observed that although procedure was quite effective in inducing primary CTL responses to peptide-pulsed target cells, only a few of the resulting CTL (~10%) were capable of killing targets that naturally produced and processed the antigen. In some instances the inability of the peptide-reactive CTL to recognize the endogenously processed antigen could be attributed to a low avidity of the CTL-target cell interaction (Wentworth, *et al.*, *Molec. Immunol. 32*:603 (1995)).

In order to optimize the *in vitro* CTL printing procedure we have evaluated the use of dendritic cells (DC) as a source of APC and the inclusion of IL-7, IL-10 and IL-12, which have been reported to participate in CTL responses (Celis, *et al.*, *Proc. Natl. Acad. Sci. (USA) 91*:2105 (1994); Alderson, *et al.*, *J. Exp. Med. 173*:923; Kos, *et al.*, *Eur. J. Immunol. 22*:3183 (1992); Chen, *et al.*, *J. Immunol. 147*:528 (1991); Yang, *et al.*, *J. Immunol. 155*:3897 (1995); Ferrari, *et al.*, *Clin. Exp. Immunol. 101*:239 (1995);

Mehrotra, *et al.*, *J. Immunol. 151*:2444 (1993); Mehrotra, *et al.*, *J. Immunol. 154*:5093 (1995); Gately, *et al.*, *Cell Immunol. 143*:127 (1992); Chouaib, *et al.*, *Proc. Natl. Acad. Sci. (USA) 91*:12659 (1994); Fallarino, *et al.*, *J. Immunol. 156*:1095 (1996). Our results show that DC are indeed more effective than the SAC-1 generated APC in eliciting antigen-specific CTL capable of recognizing naturally processed antigen from primary lymphocyte cultures. Furthermore, the use of IL-7 and IL-10 but not IL-12 greatly enhanced the capacity of DC to trigger these CTL responses.

EXAMPLES

The following examples are offered by way of illustration, not by way of limitation.

Materials and Methods

Synthetic Peptides

Peptides were prepared on an Applied Biosystems machine (Foster City, CA). Briefly, after removal of the α-amino-*ter*-butylocarbonyl protecting group, the phenylacetamidomethyl resin peptide was coupled with a 4 fold excess of preformed symmetrical anhydride (hydroxybenzyltriazole esters for arginine, histidine, asparagine, and glutamine) for 1 hr in dimethylformamide. For arginine, histidine, asparagine, glutamine and histidine residues, the coupling step was repeated in order to obtain a high efficiency coupling. Peptides were cleaved by treatment with hydrogen fluoride in the presence of the appropriate scavengers. Synthetic peptides were purified by reverse phase high pressure liquid chromatography (RV-HPLC). The purity and identity of the peptides was determined by amino acid sequence and mass spectrometric analysis respectively, and was routinely > 95%.

MHC Binding Assays

Peptide binding assay to purified HLA-A2.1 molecules was measured as previously described. Rupert *et al.*, Cell: 74:929 (1993); Sette *et al.*, Molec. Immunol. 31: 813 (1994). Briefly, the assay is based on the inhibition of binding of radiolabeled standard peptides to detergent solubilized MHC molecules. For example, a standard peptide used for the HLA-A2.1 binding studies was FLPSDYFPSV (HBC_{18-27}), a CTL epitope from hepatitis B virus core (nucleoprotein) antigen, positions 18-27 (Bertoletti, *et al.*, *J. Virol. 67*:2376 (1996)). Peptide $MAGE3_{161}$ (EVDPIGHLY) corresponds to CTL epitope recognized in the context of the HLA-A1 allele (Celis, *et al.*, *Proc. Natl. Acad. Sci. (USA) 91*:2105 (1994); Gaugler, *et al.*, *J. Exp. Med. 179*:921 (1994)). Peptide $G9_{280}$ (YLEPGPVTA) corresponds to CTL epitope from the melanosomal gp100 molecule which is recognized by HLA-A2.1 melanoma patients (Cox *et al.*, *Science 264*:716

(1994); Kawakami, *et al.*, *J. Immunol. 154*:3961 (1995)). A standard peptide for A68.2 binding assays was FTQAGYPAL.

The standard peptide was radioiodinated using ^{125}I (ICN, Irvine, CA) by the chloramine T method. HLA-A2.1 concentration yielding approximately 15% of bound peptide (approximately in the 10 nM range) was used in the inhibition assays. Various doses of the test peptides (10 μM to 1 nM) were incubated together with 5 nM radiolabeled standard peptide and HLA-A2.1 molecules for 2 days at room temperature in the presence of a cocktail of protease inhibitors and I μM β2-microglobulin (Scripps Labs., San Diego, CA). At the end of the incubation period, the % MHC bound radioactivity was determined by gel filtration, and the concentration of the test peptides that inhibited 50% of the binding of the label peptide (IC_{50}) was calculated.

Cell Lines

The .221(A2.1) cell line was produced by transferring the HLA-A2.1 gene into the HLA-A, -B, -C-null mutant human B lymphoblastoid cell line .221 (Celis, E. *et al.*, Proceedings of the National Academy of Sciences of the United States of America 91:2105-2109 (1994)). Flow cytometry of anti-class I MHC antibody binding showed that .221(A2.1) cells express 50-100 % as much as cell surface HLA-A molecules as do normal lymphoblastoid cells expressing single copies of the same HLA alleles in their native chromosomal locations. The .221(A2.1) cells express 50-100% as much as cell surface HLA-A molecules as do normal lymphoblastoid cells as determined by flow cytometry using anti-class I MHC specific antibodies. The .221(A2.1) cell line was transfected with the gene coding for the hepatitis B virus core (nucleoprotein) antigen as described (Wentworth, *et al.*, *Molec. Immunol. 32*:603 (1995)).

The Steinlin EBV-LCL (HLA-A1/1, B8/8) was used to test CTL recognition of peptide in the context of HLA-A1 molecules.

The HLA-typed melanoma cell lines used in these studies (624mel (A2.1, A3, B7, B14, gp100$^+$); A375mel (A1, A2.1, B17, gp100$^-$); 938mel (A1, A24, B7, B8, *MAGE-3*$^+$); 888mel (A1, A24, B52, B55, *MAGE-3*$^-$) have been previously described. Kawakami *et al.*, Proc. Natl. Acad. Sci. 91: 6458 (1994); Kawakami *et al.*, J. Immunol. 154: 3961 (1995). The colon adenocarcinoma cell lines SW403 and HT-29 were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in

culture as recommended by the supplier. The gastric cancer cell line KATO-III was obtained from the Japanese Cancer Research Resources Bank. All tumor lines were maintained in culture using RPMI-1640 medium supplemented with antibiotics and 10% (V/V) FBS (and 400 μg/ml G418 for the .221(A2.1) cell lines). Melanoma colon and gastric cancer cells were treated with 100 U/ml γ-IFN (Genzyme) for 48 hr at 37°C before testing their susceptibility to lysis by the CTL. In all cases, the γ-IFN-treated cells increased the level of MHC class I expression by 2-3 fold (unpublished). Although γ-IFN treatment increased the levels of CTL mediated cytotoxicity, untreated melanoma cells were still recognized by the CTL (data not shown).

Expression of *MAGE2* and *MAGE3* was determined by RT-PCR as described (De Smet, C. *et al.*, *Immunogenetics* 39:121-129 (1994); Gaugler, B. *et al.*, *J Exp Med.* 179:921-930 (1994); Zakut, R. *et al.*, *Cancer Research* 53:5-8 (1993)). In addition, the expression of *MAGE3* protein was determined by immunohistochemistry using specific monoclonal antibodies (Kocher, T. *et al.*, *Cancer Research* 55:2236-2239 (1995)). The expression of CEA and HER2/*neu* was measured by cytofluorometric analysis using the monoclonal antibodies CEMO1O (Takara Shuzo, Japan) and c-neu Ab-5 (Oncogene Science, Uniondale, NY) respectively.

Purification of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) are isolated from an HLA-typed patient by either venipuncture or leukapheresis (depending upon the initial amount of CTLp required), and purified by gradient centrifugation using Ficoll-Paque (Pharmacia). Typically, one can obtain one million PBMC for every ml of peripheral blood, or alternatively, a typical leukapheresis procedure can yield up to a total of 1-10 X 10^{10} PBMC.

For example, peripheral blood mononuclear cells (PBMC) from HLA-A2.1^{+} normal volunteers and a melanoma patient were purified by centrifugation in Ficoll-Paque (Pharmacia, Piscataway, NJ) from leukopheresis products. PBMC were also obtained from a 51 year old male patient diagnosed with cutaneous melanoma. A total of 18 out of 43 nodes were found positive for tumor cells.

The isolated and purified PBMC are co-cultured with an appropriate number of APC expressing empty MHC molecules, previously incubated ("pulsed") with

an appropriate amount of synthetic peptide (containing the HLA binding motif and the sequence of the antigen in question). PBMC are usually incubated at 1-3 X 10^6 cells/ml in culture medium such as RPMI-1640 (with autologous serum or plasma) or the serum-free medium AIM-V (Gibco).

In Vitro Generation of Dendritic Cells

APC are usually used at concentrations ranging from 1 X 10^4 to 1 X 10^6 cells/ml, depending on the type of cell used. Possible sources of APC include: autologous PBMCs, SAC-I activated PBMCs, PHA blasts; autologous dendritic cells (DC) which are isolated from PBMC and purified as described (Inaba, *et al.*, *J. Exp. Med.*, 166:182 (1987)); and mutant and genetically engineered mammalian cells such as the mouse RMA-S cell line or the human T2 cell line transfected with the appropriate MHC genes that express "empty" HLA molecules which are syngeneic to the patient's allelic HLA form). APC containing empty HLA molecules are known to be potent inducers of CTL responses, possibly because the peptide can associate more readily with empty MHC molecules than with MHC molecules which are occupied by other peptides (DeBruijn, *et al.*, *Eur. J. Immunol.*, 21:2963-2970 (1991)).

Tissue culture-derived dendritic cells (DC) were used as APC to trigger CTL responses. Dendritic cells (DC) were generated from monocyte-enriched cell fractions as described (Tsai *et al.*, *Cell. Immunol. 144*:203 (1992)) or by adherence to plastic (2 hr at 37°C in serum-containing medium). Briefly, PBMC were separated on multi-step Percoll gradients (30, 40, 50.5 and 70%) and the monocyte-enriched population was harvested in the light density fraction at the 40-50.5% interface. The washed monocytes containing the DC precursors were cultured at 5 X 10^5 cells/ml in the presence of 50 ng/ml of GM-CSF and 1,000 U/ml of IL-4 (both from R&D Systems, Minneapolis, MN) as described in Romani, *et al.*, *Exp. Med.* 180:83 (1994); Sallusto, *et al.*, *J. Exp. Med. 179*:1109 (1994), in complete medium (see below). The adherent monocyte population was treated in the same manner. After 7 days the cells, which display typical cell surface markers of DC ($CD3^-$, $CD14^-$, $CD88^+$, HLA-A,-B,-C^{HI}, $HLA\text{-}DQ^+$; data not shown) were harvested.

"Fresh" DC were isolated directly from a pheresis product as described (Tsai, *et al.*, *J. Clin. Invest 82*:1731 (1988)). Briefly, monocytes and DC were first

purified with a Percoll gradient as described above and the majority of monocytes were removed by adherence to plastic for 2 hr at 37°C. The DC fraction, which is released from the substrate is further purified by eliminating contaminating monocytes and lymphocytes using a single step Metrizamide gradient. Between 50 and 70% of the resulting cells have typical DC markers and can be distinguished from other cell types in phase contrast microscopy.

CTL Induction Using Synthetic Peptides and Dendritic Cells

CTL lines were expanded in tissue culture following a method similar to the one described by Riddell, *et al.*, *Nature Medicine* 2:216 (1996)) for CTL clones. A total of 5 X 10^4 CTL were resuspended in 25 ml of complete medium with 25 X 10^6 autologous irradiated (4200 rads) PBMC (The APC are gamma irradiated to prevent their proliferation and to facilitate the expansion of the CTLp.) and 5 X 10^6 allogeneic (non HLA-A2) and irradiated (8000 rads) Epstein-Barr virus-transformed lymphoblastoid B-cells in the presence of 30 ng/ml of anti-CD3 monoclonal antibodies (OKT3). CTL lines were also expanded using peptide-pulsed PBMC (instead of anti-CD3 antibodies), in order to maintain antigen specificity.

Mixed PBMC, APC and peptide were kept in an appropriate culture vessel such as plastic T-flasks, gas-permeable plastic bags, or roller bottles, at 37° centigrade in a humid air/CO_2 incubator. One day after initiating the cultures, 200 IU/ml of recombinant IL-2 were added to the cultures. The cultures were fed with fresh medium containing 50 IU/ml IL-2 on days 5, 8 and 11 and were split if the T-cell concentration reached numbers > 1.5 X 10^6/ml.

After the activation phase of the culture, which usually occurs during the first 3-5 days, the resulting effector CTL can be further expanded, by the addition of recombinant growth factors such as interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-7 (IL-7), or interleukin-10 (IL-12) to the cultures. An expansion culture can be kept for an additional 5 to 12 days, depending on the numbers of effector CTL required for a particular patient. In addition, expansion cultures may be performed using hollow fiber artificial capillary systems (Cellco), where larger numbers of cells (up to $1X10^{11}$) can be maintained.

In order to obtain the required cell numbers for treatment, it may be necessary to restimulate the cultures 2-4 times with irradiated, autologous, peptide pulsed adherent PBMCs. On the average, approximately 5 X 10^7 CTL were obtained by day 14-16.

Aliquots of 1-2 X 10^6/ml DC were separately pulsed for 4 hours at 20°C with 40 μg/ml of peptide in the presence of 3 μg/ml of β_2 microglobulin and were irradiated (4,200 rad) before used. $CD8^+$ T-cells purified with immunomagnetic beads (Dynabooks M-450 and Detachabend; Dynal) were used as responders. Typically, to obtain sufficient cells for a 48-well plate culture, 200~250 X 10^6 PBMC were processed to obtain 24 X 10^6 $CD8^+$ T cells. CTL priming cultures were set up in 48-well tissue culture plates (Costar) by mixing 0.25 ml cytokine-generated DC (at 1 X 10^5 cells/ml) with 0.25 ml of $CD8^+$ T-cells (at 20 X 10^5 cell/ml) in each well in the presence 10 ng/ml of IL-7 (Genzyme). 24 hrs. after the cultures were initiated, 10 ng/ml of recombinant IL-10 (Endogen, Cambridge, MA) were added to each well. CTL cultures (each individual well) were re-stimulated every 7 days (up to 6 cycles) with irradiated (3,300 rad) autologous monocytes pulsed with peptide as described. Recombinant IL-10 (10 ng/ml) and recombinant IL-2 (10 IU/ml) were added 1 day and 2 days respectively after each re-stimulation cycle. For one experiment (Table II), medium supernatants were sampled (100 μ1) just prior the second antigen restimulation cycle and 24 hours after antigen restimulation, to determine the production of GM-CSF, which was measured using an ELISA kit (R&D Systems, Minneapolis, MN). Complete culture medium consisted of RPMI- 1640 (Gibco, Grand Island, NY) supplemented with 5% AB male human serum, L-Glutamine, sodium pyruvate non-essential amino acids (all from Gibco) at the recommended concentrations.

CTL Cytotoxicity Assays

Before expanded cells are used (*e.g.*, infused into a patient), they are tested for activity, viability, toxicity and sterility.

The cytotoxic activity of the resulting CTL can be determined by a standard ^{51}Cr-release assay (Biddison, W.E. (1991), Current Protocols in Immunology, p7,17.1-7.17.5, Ed. J. Coligan *et al.*, J. Wiley and Sons, New York), using target cells that express the appropriate HLA molecule, in the presence and absence of the

immunogenic peptide. For example, peptide-pulsed targets were prepared incubating the cells at about 5 X 10^5 cells/ml with 10 μg/ml synthetic peptide overnight at 37°C. All cells were labeled with 100-300 μCi ^{51}Cr(ICN) per 3 X 10^6 cells for 1 hr at 37°C. Adherent target cells (*e.g.*, melanomas) were detached from tissue culture flasks with Trypsin-EDTA. Target cells were washed by centrifugation and mixed with effectors in a final volume of 0.2 ml of RPMI-1640 containing 10% fetal bovine serum (FBS, GIBCO) in round-bottom microtiter plates. The plates were centrifuged (5 min at 400 x g) to increase cell to cell contact and placed in a 37°C CO_2 incubator. After 4-6 hr incubation time, and 0.1 ml of the supernatant was removed from each well, and the radioactivity was determined in a γ-counter. In the initial screening for the presence of cytolytic activity in each well of the 48-well minicultures, the effector cells were routinely not counted. However, sample counting of these cells indicated that effector:target ratios in these assays were in the range of 10:1 to 1:1. Antigen specificity was determined by cold target inhibition analyses by determining the capacity of peptide pulsed unlabelled .221 (A2.1) cells to block the lysis of melanoma cells.

% Specific Cytotoxicity was determined by calculating the % Specific ^{51}Cr release by the formula: [(cpm of the test sample - cpm of the spontaneous ^{51}Cr release)/(cpm of the maximal ^{51}Cr release - cpm of the spontaneous ^{51}Cr release)] x 100. The spontaneous ^{51}Cr release was determined by incubating the targets alone, in the absence of effectors, and the maximal ^{51}Cr release was obtained by incubating the targets with 0.1% Triton X-100 (Sigma Chem. Co., St. Louis, MO). Determinations were performed in duplicate. The standard error of the means were typically below 10% of the value of the mean.

Viability was determined by the exclusion of trypan blue dye by live cells. Cells are tested for the presence of endotoxin by conventional techniques. Finally, the presence of bacterial or fungal contamination was determined by appropriate microbiological methods (chocolate agar, *etc.*). Once the cells pass all quality control and safety tests, they are washed and placed in the appropriate infusion solution (Ringer/glucose lactate/human serum albumin) which may include a T-cell growth factor such as IL-2 and infused intravenously into the patient.

EXAMPLE 1

Preparation of effective HLA allele-specific antigen presenting cells by acid stripping followed by peptide loading.

This example demonstrates the use of cold temperature incubation and acid stripping for generation of empty MHC class I molecules to enable peptide loading method to prepare effective HLA-allele-specific antigen presenting cells (APC) for use in diagnostic or *ex vivo* therapy applications. A similar method is described in related U.S. application Serial No. 08/468,454, filed on June 6, 1995, which is a continuation of U.S. application Serial No. 08/103,401, filed on August 6, 1993, both of which are incorporated by reference. The APC in this example were used to sensitize precursor cytotoxic T lymphocytes for the development of antigen-specific cytotoxic cells. This was accomplished using either staphylococcus aureus cowan I SAC I activated PBMC, phytohemagglutinin (PHA) T-cell blasts or peripheral blood mononuclear cells (PBMC) as APC in the HLA-A2.1 and HLA-A1 systems. The results are applicable to other APC and to the other MHC alleles.

Culture Medium.

PHA blasts and CTL inductions were done in RPMI 1640 + Hepes + glutamine (Gibco) supplemented with 2 mM L-glutamine (Irvine Scientific), 50 μg/ml gentamicin (Gibco), and 5% heat inactivated pooled human Type AB serum (Gemini Bioproducts) [RPMI/5% HS]. EBV transformed lymphoblastoid cell lines (LCL) were maintained in RPMI 1640 + Hepes + glutamine (BioWhittaker) supplemented with L-glutamine and gentamicin as above and 10% heat inactivated fetal calf serum (Irvine Scientific) [RPMI/10% FCS]. Chromium release assays were performed in RPMI/10% FCS.

Cultured Cell Lines.

JY, a HLA A2.1 expressing human EBV-transformed B-cell line, was grown in RPMI/10% FCS. K562, a NK cell sensitive erythroblastoma line was grown in RPMI/10% FCS. K562 was used to reduce background killing by NK and LAK cells in the chromium release assays.

Peptides.

The immunogenic peptides used in these studies were synthesized as described above using motifs for HLA alleles for specific target antigens as described in copending commonly assigned applications U.S.S.N. 07/926,666 and U.S.S.N. 08/027,146. Their sequences are shown in Table I. Peptides were routinely dissolved in 100% DMSO at 20 mg/ml, aliquoted, and stored at -20°C.

Isolation of Peripheral Blood Mononuclear Cells (PBMC).

Whole blood was collected in heparin (10 U/ml) containing syringes and spun in 50cc conical centrifuge tubes (Falcon) at 1600 rpm (Beckman GS-6KR) 15 min. The plasma layer was then removed and 10 ml of the buffy coat collected with a 10 ml pipette using a circular motion. The buffy coat was mixed thoroughly and diluted with an equal volume of serum free RPMI 1640. The diluted buffy coat was then layered over 20 ml Ficoll-Paque (Pharmacia) in a 50cc conical tube and centrifuged 400xg for 20 minutes at room temperature without the brake off. The interface containing the PBMCs was collected using a transfer pipet (two interfaces per 50cc tube) and washed three times with 50 ml serum free RPMI (1700, 1500, and 1300 rpm for 10 minutes.

Freezing and Thawing PBMC.

PBMC were frozen at 30 x 10^6 cells/ml of 90% FCS + 10% DMSO (Sigma) in 1 ml aliquots using cryovials (Nalge). Cryovials were placed in Cryo 1°C freezing containers (Nalge) containing isopropanol (Fisher) and placed at -70°C from 4 hours (minimum) to overnight (maximum). Isopropanol was changed after every 5 uses. Cryovials were transferred to liquid nitrogen for long term storage. PBMC were thawed by continuous shaking in a 37°C water bath until the last crystal was nearly thawed. Cells were immediately diluted into serum free RPMI medium containing DNAse 30 μg/ml (to avoid clumping by dead cells) (Calbiochem) and washed twice.

Preparation of CD4+ T cell depleted responder cell population.

CD4+ lymphocyte depletion was performed using antibody-coated flasks: MicroCELLector T-150 flasks for the selection of CD4+ cells (Applied Immune Sciences) were washed according to the manufacturer's instructions with 25 ml PBS CMF

(calcium magnesium free) + 1 mM EDTA (Sigma) by swirling flasks for 30 sec followed by incubation for 1 hour at room temperature on a flat surface. Buffer was aspirated and flasks were washed 2 additional times by shaking the flasks for 30 seconds and maintaining coverage of the binding surface. To each washed flask, 25 ml culture medium were added and incubated for 20 minutes at room temperature on a flat surface. Media was left in the flask until it was ready to receive the cells. PBMC were thawed in culture medium containing 30 μg/ml DNAse and washed twice. For one flask a maximum of 12 x 10^7 cells were resuspended in 25 ml culture medium. Culture medium was aspirated from the flask and then the cell suspension was gently added to the MicroCELLector. Flasks containing the cells were incubated for 1 hour at room temperature on a flat surface. At the end of the incubation, the flask was gently rocked from side to side for 10 seconds to resuspend the nonadherent cells. Nonadherent CD4+ T cell depleted cells were harvested and then flasks were washed twice with PBS CMF to collect the nonadherent cells. Harvested CD4+ T cell depleted cells were pelleted by centrifugation and resuspended in culture medium.

Generation of PHA Blasts.

PBMC were isolated using the standard Ficoll-Paque protocol. Frozen cells were washed twice before use. Cells were cultured at 2 x 10^6/ml in RPMI/5% HS containing 1 μg/ml PHA (Wellcome) and 10 U/ml rIL-2. PHA blasts were maintained in culture medium containing 10 U/ml rIL-2 with feeding and splitting as needed. PHA blasts were used as APCs on day 6 of culture. Generation of empty class I molecules and peptide loading was only performed by the acid strip method when using PBMCs as APCs.

Acid Stripping/Peptide Loading of PBMC and PHA Blasts.

PBMC were isolated using the Ficoll-Paque protocol. When using frozen cells, PBMC were washed twice before using. PHA blasts were prepared as previously described and washed twice before using. Once cells were prepared, they were washed once in cold sterile 0.9% NaCl (J.T. Baker) + 1% BSA. In a 50cc conical centrifuge tube, the cells were resuspended at 10^7/ml in cold sterile citrate-phosphate buffer [0.13 M citric acid (J.T. Baker), 0.06 M sodium phosphate monobasic (Sigma) pH 3, 1% BSA, 3

μg/ml β_2 microglobulin (Scripps Labs)] and incubated for 2 minutes on ice. Immediately, 5 volumes of cold sterile neutralizing buffer #1 [0.15 M sodium phosphate monobasic pH 7.5, 1% BSA, 3 μg/ml β_2 microglobulin, 10 μg/ml peptide] were added, and the cells were pelleted at 1500 rpm, 5 min at 4°C. Cells were resuspended in 1 volume cold sterile neutralizing buffer #2 [PBS CMF, 1% BSA, 30 μg/ml DNAse, 3 μg/ml β_2 microglobulin, 40 μg/ml peptide] and incubated for 4 hours at 20°C. Cells were diluted with culture medium to approximately 5 x 10^6/ml and irradiated with 6000 rads. Cells were then centrifuged at 1500 rpm for 5 minutes at room temperature and resuspended in culture medium. The acid stripped/peptide loaded cells were used immediately in the CTL induction cultures (below).

Binding Assays Using Intact Cells and Radiolabelled Peptide.

JY cells were either acid stripped (i.e. treated with citrate-phosphate buffer and neutralizing buffer #1 as described above) or incubated at a reduced temperature. JY control cells were left untreated in tissue culture media. After treatment both cell populations were washed twice with serum free RPMI and loaded with ^{125}I-radiolabelled 941.01 (HBc 18-27) peptide (standard chloramine T iodination). To determine binding specificity, 2 x 10^6 cells were resuspended in 200 μl neutralizing buffer #2 (described above) containing ^{125}1-941.01 (10^5 cpms) +/- 100 μg unlabelled 941.01. Cells were incubated for 4 hours at 20°C and washed twice with serum free RPMI to remove free peptide. Cells were resuspended in 200 μl of serum free RPMI. In a microfuge tube the cell suspension was layered over an 800 μl FCS and pelleted by centrifugation for 5 seconds. Supernatants were aspirated and the radioactivity remaining in the pellet was measured (Micromedic automatic gamma counter, 1 minutes per tube).

Binding of Radiolabeled Peptides to Empty MHC Molecules.

To determine the efficiency of peptide loading using the cold temperature incubation or acid stripping peptide loading protocol, JY cells (an HLA-A2.1 EBV-transformed B cell line) were preincubated at 26°C overnight or acid-stripped to remove the endogenous MHC-associated peptides and the loading of exogenous peptide was determined using a ^{125}I-radiolabelled HLA-A2.1 binding peptide. The specificity of this reaction was determined by measuring the inhibition of labelled peptide binding using

a cold peptide of the same sequence. Results presented in Table 2 demonstrate that acid-treatment of the cells increased significantly (approximately 10-fold) the amount of labelled peptide binding to the JY cells. Furthermore, the binding of labelled peptide was completely blocked by the addition of the cold peptide, demonstrating specific binding (data not shown).

FACS Analysis.

Approximately 10^6 cells were used for each antibody that was to be tested. Cells were washed twice with PBS CMF + 0.1% BSA. To each sample, 100 μl PBS CMF + 0.1% BSA + primary antibody at 2 μg/ml (BB7.2, ATCC) or (9.12.1, INSERM-CNRS, Marseille) or (LB3.1, Children's Hospital, Pittsburgh) were added. A negative control was always included. Cells were incubated on ice for 20 minutes and washed twice with PBS CMF + 0.1% BSA. Cells were resuspended in 100 μl anti-mouse IgG FITC conjugate (Sigma), diluted 1:50 in PBS CMF + 0.1% BSA, and incubated 20 minutes on ice. Cells were washed twice with PBS CMF + 0.1% BSA, and resuspended in PBS for FACScan (Becton Dickinson) analysis. When it was necessary to postpone analysis to the subsequent days, the cells were fixed with PBS/1% paraformaldehyde (Fisher) and analyzed within one week.

Measurements by FACS Analysis.

PHA-induced T-cell blasts were acid stripped/peptide loaded according to the methods described above. The resulting cells were stained for FACS analysis using anti-HLA-A2 (BB7.2) and anti-HLA alpha chain-specific (9.12.1) monoclonal antibodies. Controls for this experiment included the same cell population which was not treated at pH 3 (but treated with PBS buffer at pH 7.2), and cells treated with citrate-phosphate buffer (to strip the MHC) but neutralized in the absence of β_2 microglobulin and peptide. The results indicate that treatment of these cells with the citrate-phosphate (pH3) buffer significantly reduced (10-fold) the reactivity of the cells toward both anti-HLA class I antibodies alone (anti-HLA-A2 and the alpha chain specific), but not towards a monoclonal antibody specific for class II MHC molecules (anti-HLA-DR). Most importantly, neutralization of the acid-stripped cells in the presence of β_2 microglobulin and peptide resulted in preservation of a significant amount of class I MHC

antibody-reactive sites, with only a 2.5-fold decrease in fluorescence intensity. The acid-treated cells remained viable, as measured by trypan blue exclusion and forward/lateral FACS scatter analysis. Similar results were obtained using EBV-transformed B cell lines, fresh (or frozen) PBMC and other peptides (which bind to either HLA-A2.1 or HLA-Al) (data not shown).

Induction of Primary CTL using Acid Stripped/Peptide Loaded Autologous PBMCs or PHA Blasts as Stimulators.

Acid stripping/peptide loading of PBMC and PHA blasts are described above. During the 4 hour incubation of stimulator cells with peptide, the responder cell population was prepared: Responders were PBMC that were depleted of CD4+ T cells (described above). Responder cells were resuspended in culture medium at 3 x 10^6/ml and 1 ml of the responder cell suspension was dispensed into each well of a 24-well tissue culture plate (Falcon, Becton Dickinson). The plates were placed in the incubator at 37°C, 5% $C0_2$ until the stimulator population was ready. Once irradiated, stimulator APCs were resuspended in culture medium containing 20 ng/ml rIL-7 at 10^6/ml for the PBMC, or at 3 x 10^5/ml for the PHA blasts, 1 ml of stimulator cell suspension was added per well to the plates containing the responders. On day 7 after induction, 100 μl culture medium containing 200 ng/ml rIL-7 was added to each well (10 ng/ml rIL-7 final). On day 10 after induction, 100 μl of culture medium containing 200 U/ml rIL-2 was added to each well (10 U/ml rIL-2 final).

Additional critical parameters for the induction of primary CTL are: 1) enrichment of CD8+ T-cells in the responder cell population (by depletion of CD4+ T-cells), 2) addition of rIL-7 to the CTL induction cultures from day 0, and 3) restimulation of the cultures with antigen on day 12-14 using autologous adherent cells pulsed with peptide.

Antigen Restimulation of CTL.

On day 12-14 after the induction, the primary CTL were restimulated with peptide using autologous, adherent APCs. Autologous PBMC were thawed and washed as described above. Cells were irradiated at 6000 rads. Cells were pelleted and resuspended in culture medium at 4 x 10^6/ml and 1 ml of cell suspension was added to

each well of a 24-well tissue culture plate, and incubated for 2 hours at 37°C, 5% CO_2. Nonadherent cells were removed by washing each well three times with serum free RPMI. After this step, a 0.5 ml culture medium containing 3 μg/ml $\beta_2$microglobulin and 20 μg/ml total peptide was added to each well. APC were incubated for 2 hrs at 37°C, under 5% CO_2 with the peptide and $\beta_2$microglobulin. Wells were aspirated and 1 ml of responder cells at 1.5 x 10^6/ml in culture medium was added to each well. After 2 oays, 1 ml of culture medium containing 20 U/ml rIL-2 was added to each well. Cultures were supplemented with 10 U/ml rIL-2 (final) every three days thereafter.

Cytotoxicity Chromium Release Assay.

Seven days following restimulation of primary induction, the cytotoxic activity of the cultures was assessed.

a. Effector Cell Preparation: The responders were centrifuged and resuspended at 10^7/ml in RPMI/10% FCS. Three-fold serial dilutions of effectors were performed to yield effector to target ratios of 100:1, 33:1, 11:1, and 3:1. Effector cells were aliquoted at 100 μl/well on 96 well U-bottomed cluster plates (Costar), in duplicate.

b. Target Cell Preparation: Approximately 16-20 hours prior to the assay, target cells were resuspended at 3 x 10^5/ml in RPMI/10% FCS in the presence or absence of 3 μg/ml $\beta_2$microglobulin and 10 μg/ml total peptide. After preincubation, target cells were centrifuged and pellets were resuspended in 200 μl (300μCi) sodium (^{51}Cr) chromate (NEN). Cells were incubated at 37°C for 1 hour with agitation. Labelled target cells were washed 3 times with RPMI/10% FCS.

c. Setting-Up the Assays: Target cell concentration was adjusted to 10^5/ml in RPMI/10% FCS and 100 μl aliquots were added to each well containing responders. K562 cells (cold targets, to block NK, and LAK activity) were washed and resuspended in RPMI/10% FCS at 10^7/ml. Aliquots of 20 μl were added per well, yielding a 20:1 cold K562 target to labelled target ratio. For the determination of the spontaneous ^{51}Cr release, 100 μl/well of RPMI/10% FCS were added to 100 μl/well of labelled target cells, and 20 μl/well of K562. For maximum ^{51}Cr release, 100 μl 1% Triton X-100 (Sigma) in PBS CMF, was added to the 100 μl/well labelled target cells, and 20 μl/well K562. Plates were centrifuged for 2 minutes at 1200 rpm to accelerate cell conjugate formation. Assays were incubated for 5 hours at 37°C, 5% $C0_2$. Assays

were harvested by centrifuging plates for 5 minutes at 1200 rpm and collecting 100 μl/well of supernatant. Standard gamma counting techniques were used to determine percent specific lysis (Micromedic automatic gamma counter, 0.5 minutes per tube). Percent specific lysis was determined by the following formula: cpm experimental release -cpm spontaneous release/cpm maximum release-cpm spontaneous release x 100.

EXAMPLE 2

Screening peptides to identify CTL epitopes.

In order to identify CTL epitopes, CTL were stimulated by SAC-I activated PBMCs as APC. Cold temperature enhanced expression of empty MHC enabling loading of antigenic peptide to generate SAC-I activated PBMC APC. This method presents an alternative protocol to the methods described above for the generation of the APC which are used to stimulate CTL. This example also presents an alternative protocol for the stimulation of CTL by the APC.

Complete Culture Medium.

The tissue culture medium used in this study consisted of RPMI 1640 with Hepes and L-glutamine (Gibco) (Biowhittaker) supplemented with 2 mM L-glutamine (Irvine Scientific), 0.5 mM sodium pyruvate (Gibco), 100 U/100 ug/ml penicillin/streptomycin (Irvine), and 5% heat-inactivated Human Serum Type AB (RPMI/5% HS; Gemini Bioproducts). Culture media used in the growth of EBV-transformed lines contained 10% heat-inactivated fetal calf serum (RPMI/10% FCS, Irvine) instead of human serum.

Cytokines.

Recombinant human Interleukin-2 (rIL-2) and Interleukin-4 (rIL-4) were obtained from Sandoz and used at a final concentration of 10 U/ml and 10 ng/ml, respectively. Human interferon-γ (IFN-γ) and recombinant human Interleukin-7 (rIL-7) were obtained from Genzyme and used at 20 U/ml and 10 ng/ml, respectively.

Peptides. Peptides were synthesized as described above and are described in Table I. Peptides were routinely dissolved in 100% DMSO at 20 mg/ml, aliquoted, and stored at -70°C until used.

Cell Lines.

JY, Steinlein, EHM, BVR, and KT3 are homozygous human EBV-transformed B cell lines expressing HLA $A_{2.1}$, A_1, A_3, A_{11}, and A_{24}, respectively. They are grown in RPMI/10% FCS and used as targets in the CTL assays. K562, an NK cell sensitive, erythroblastoma line grown in RPMI/10% FCS, was used for reduction of background killing in the CTL assays. Melanoma HLA A1+ cell lines either expressing the MAGE antigen, mel 397 and mel 938 or those not expressing the MAGE antigen, mel 888, were also grown in RPMI/10% FCS.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs).

Whole blood was collected into heparin containing syringes and spun in 50cc tubes at 1600 RPM (Beckman GS-6KR) for 15 minutes. The plasma layer was then removed and 10 ml of buffy coat was collected with a pipette using a circular motion. The buffy coat was mixed well and diluted with an equal volume of RPMI. The buffy coat (30 ml) was then layered on 20 ml of Ficoll-Paque (Pharmacia) and centrifuged at 1850 RPM (400xg) for 20 minutes, 25°C, with the brake off. The interface between the Ficoll and the plasma containing the PBMCs was recovered with a transfer pipet (two interfaces per 50 ml tube) and washed three times with 50 ml of RPMI (1700, 1500, and 1300 RPM for 10 minutes). Cells were resuspended in 10-20 ml of culture medium, counted, and adjusted to the appropriate concentration.

Freezing PBMCs.

30 million cells/tube (90% FCS/10% DMSO; Sigma) were inserted into a Nalgene Cryo 1°C Freezing Container containing isopropanol (Fisher) and placed at -70°C from 4 hrs (minimum) to overnight (maximum). The isopropanol was changed every five times. Tubes were transferred to liquid nitrogen for long term storage. To thaw, PBMCs were continuously shaken in a 37°C water bath until the last crystal was almost thawed (tubes were not allowed to sit in the water bath or at room temperature for

any period of time). Cells were diluted into serum-free RPMI containing 30 μg/ml DNase to prevent clumping by dead cell DNA and washed twice.

Induction of Primary CTL Using SAC-I Activated PBMCs as APCs

a. Preparation of SAC-I activated PBMCs as APCs:

PBMCs were purified using the standard Ficoll-Paque protocol and resuspended at 1 x 10^6/ml in RPMI/5% FCS containing 0.005% Pansorbin cells (SAC-I cells expressing Protein A; Calbiochem), 20 μg/ml Immunobeads (Rabbit anti-Human IgM; Biorad), and 20 ng/ml of human rIL-4. Two ml of cells per well were plated in a 24-well plate (Falcon, Becton Dickinson) and cultured at 37°C. After 3 days, the medium was removed and the cells were washed three times followed by addition of RPMI/10% HS. The cells were used after culturing for an additional 2 days in RPMI/10% HS.

b. Expression of empty Class I molecules on the surface of APCs and peptide loading of APCs.

1. Cold temperature incubation:

a. Expression of empty MHC in APCs: The APCs were adjusted to a concentration of 2 x 10^6/ml in complete culture medium containing 10 ng/ml rIL-4, 20 U/ml human IFN-γ, and 3 μg/ml β2-microglobulin (β_2m; Scripps Lab). The cells were then incubated overnight at 26°C in the presence of 5% CO_2. It should be noted that these cells only express a fraction of Class I molecules in the empty state (~10%).

b. Peptide loading of APC stimulator cells:

Empty Class I expressing APCs were washed 1-2 times with serum free RPMI (+ L-glutamine and Hepes) and resuspended at 1 X 10^7 in serum-free RPMI containing 50 μg/ml total of the peptide pool (i.e., 16.7 μg/ml of each peptide in a pool of three; 25 μg/ml of each peptide in a pool of two; 50 μg/ml of individual peptide), 30 μg/ml DNAse, and 3 μg/ml β_2m. Following a 4 hour incubation at 20°C, the cells were irradiated at 6100 rads (5 x 10^6/ ml; 25 million cells/tube), washed and adjusted to the appropriate concentration for addition to the induction culture (see below).

2. Acid stripping: This was used as an alternative method for generating empty MHC on the surface of the APCs. The SAC-I activated PBMCs were

washed once in cold 0.9% sodium chloride (J.T. Baker) containing 1% BSA. The cells were resuspended at 10^7/ml in cold citrate-phosphate buffer (0.13M citric acid [J.T. Baker], 0.06M sodium phosphate monobasic [Sigma], pH 3) containing 1% BSA and 3 μg/ml β_2m and incubated on ice. After 2 minutes, 5 volumes of cold 0.15 M sodium phosphate buffer, pH 7.5, containing 1% BSA, 3 μg/ml β_2m, and 10 μg/ml peptide [neutralizing buffer #1] was added and the cells centrifuged at 1500 RPM for 5 minutes at 4°C. The cells were resuspended in 1 ml of cold PBS containing 1% BSA, 30 μg/ml DNase, 3 μg/ml β_2 microglobulin, and 50 μg/ml peptide [neutralizing buffer #2] and incubated for 4 hours at 20°C. As above, subsequent to the four hour incubation at 20°C, the cells were irradiated at 6100 rads (5 x 10^6/ ml; 25 million cells/tube), washed, then adjusted to the appropriate concentration for addition to the induction culture (see below).

Preparation of the CD4+ depleted PBMC responder cell population (depletion of lymphocyte subpopulations using AIS flasks).

AIS MicroCellector T-150 flasks (specific for the depletion of CD4+ T cells; Menlo Park, CA) were primed by adding 25 ml of PBS/1 mM EDTA, swirling for 30 seconds so that all surfaces were moistened, and then incubating with the binding surface down at room temperature for 1 hour. Following this incubation, flasks were shaken vigorously for 30 seconds, washed 1 time with PBS/EDTA, 2 additional times with PBS and then incubated with 25 ml of culture medium for 15 minutes. PBMCs were thawed in serum-free RPMI (+ L-glutamine + Hepes) containing 30 μg/ml DNAse, washed once, and incubated for 15 minutes in culture medium. Following aspiration of culture medium from the flasks, up to 180 million PBMCs were added in 25 ml of culture medium containing 30 μg/ml DNAse. After 1 hour at room temperature, the flasks were rocked gently for 10 seconds to resuspend the nonadherent cells. The nonadherent cell suspension containing the CD8+ T cells was collected and the flasks were washed 2 times with PBS. The CD4+ T cell depleted PBMCs were centrifuged and counted for addition to the induction culture. The CD4+ and CD8+ phenotype of the CD4+ depleted cell population was determined by FACS analysis (see below). In general, this technique resulted in a two-fold enrichment for CD8+ T cells with an average of approximately 40-50% CD8+ T cells and 15-20% remaining CD4+ T cells following

depletion of CD4+ T cells. Depletion of CD4+ T cells can also be accomplished by using antibody and complement methods or antibody coated magnetic beads (Dynabeads). Depletion of CD4+ T cells enriched the CTLp and removed cells which competed for cell nutrients.

Induction of primary CTL.

During the 4 hour peptide loading of the stimulator APCs, CD4+ depleted PBMC to be used as the responder population were prepared utilizing AIS flasks for selection of CD8+ T cells through the depletion of CD4+ T cells (above). The responder cells were plated at 3 x 10^6/ml in a 1 ml volume (24 well plate) and placed at 37°C until the peptide loaded stimulator APCs were prepared. The irradiated, peptide loaded APCs were washed 1 time in serum-free RPMI (+ L-glutamine and Hepes), adjusted to the appropriate concentration in complete medium, and plated into a 24 well plate at 1 ml/plate: For PBMC and SAC-I activated PBMCs as APCs 1 x 10^6 stimulator cells (1 ml volume) were plated into the wells containing the responder cells; For PHA blasts as APCs, 1 ml of 3 x 10^5/ml stimulator cells were plated in each well. A final concentration of 10 ng/ml of rIL-7 (2 ml total volume) was added. On day 7 an additional 10 μg/ml rIL-7 was added to the culture and 10 U/ml rIL-2 was added every 3 days thereafter. On day 12, the cultures were restimulated with peptide pulsed adherent cells and tested for cytolytic activity 7 days later (below).

Protocol for Restimulation of Primary CTL Using Autologous Adherent APC.

Autologous PBMCs were thawed into serum-free RPMI (+ L-glutamine and Hepes) containing 30μg/ml DNAse, washed 2 times, and adjusted to 5 x 10^6 /ml in culture medium containing DNAse. PBMCs (25 million cells/tube in 5 ml) were irradiated at 6100R. After 1 wash, the PBMCs were resuspended in culture medium and adjusted to 4 x 10^6/ml and 1 ml of irradiated PBMCs was added per well of a 24-well plate. The PBMC were incubated for 2 hours at 37°C, washed 3 times to remove nonadherent cells, and cultured in medium containing 20 μg/ml total peptide and 3 μg/ml β_2 microglobulin added in a 0.5 ml volume and again incubated for 2 hours at 37°C. The peptide was aspirated and 1.5 x 10^6 responder cells resuspended in culture medium were

added in a 1 ml volume. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added.

FACS Analysis.

One million cells/tube were centrifuged, resuspended in 100 μl/tube PBS/0.1% BSA/0.02% sodium azide (Sigma) plus 10 μl/tube directly conjugated antibody (Becton Dickinson), and incubated on ice 15-20 minutes. Cells were then washed 2 times with PBS/0.1% BSA/0.02% sodium azide and resuspended in PBS to analyze on FACScan (Becton Dickinson). When it was not possible to analyze samples within 1-2 days, cells were fixed with PBS containing 1% paraformaldehyde (Fisher) and analyzed within one week.

Cytotoxicity Assay

a. Target cell preparation. Approximately 16-20 hours prior to the CTL assay, target cells (Class I matched EBV-transformed lines) were washed once and resuspended in a 10 ml volume at 3 x 10^5/ml in RPMI/5% FCS in the presence or absence of 10 μg/ml total peptide.

b. Labeling of target cells: Target cells were centrifuged and resuspended in 200 μl/tube sodium ^{51}Cr chromate (NEN), then incubated at 37°C for 1 hour on a shaker. Targets were washed 3 times (10 ml/wash) with RPMI/10% FCS and resuspended in 10 ml (to determine the efficiency of labelling, 50 μl/target was counted on the Micromedic automatic gamma counter).

c. CTL assay. Target cells were adjusted to 2 x 10^5/ml and 50 μl of the cell culture was added to each well of a U-bottomed 96-well plate (Costar Corp.) for a final concentration of 1 X 10^4/well. K562 cells were washed once, resuspended at 4 x 10^6/ml, and 50 μl/well was added for a final concentration of 2 x 10^5/well (ratio of cold K562 to target was 20:1). Responder cells were washed once, resuspended at 9 x 10^6/ml, and three fold serial dilutions were performed for effector to target ratios of 90:1, 30:1, 10:1, and 3:1. Responder cells were added in a volume of 100 μl in duplicate wells. For spontaneous release, 50 μl/well of labelled target cells, 50 μl/well K562, and 100 μl/well of medium was added. For maximum release, 50 μl/well target, 50 μl/well K562, and 100 μl/well of 0.1% Triton-X100 (Sigma) was added. Plates were centrifuged for 5

minutes at 1200 RPM. Following a 5 hour incubation at 37°C, plates were centrifuged again for 5 minutes at 1200 RPM, and 100 μl/well of supernatant was collected. Standard gamma counting techniques (Micromedic automatic gamma counter; 0.5 minutes/tube) were used to determine the percent specific lysis according to the formula: % specific lysis = cpm experimental release - cpm spontaneous release/cpm maximum release - cpm spontaneous release X 100. A cytotoxicity assay (CTL assay) was considered positive if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T) ratios was 15% greater than lysis of control targets (i.e. target cells without peptide). A cytotoxicity assay (CTL assay) was considered borderline if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T) ratios was 6% greater than lysis of control targets (i.e. target cells without peptide).

Results

Of the peptides that bind to the indicated alleles, 12 of the 60 MAGE peptides, 13 of the 53 HIV peptides, 3 of the 25 HCV peptides, and 7 of the 28 HBV peptides tested to date induced primary CTL *in vitro*.

EXAMPLE 3

Induction of CTL responses using peptide-pulsed dendritic cells together with IL-7 and IL-10 to stimulate CTL expansion

We have demonstrated that SAC-1 stimulated PBMC functioned effectively as APC for generating CTL responses to a high affinity MHC binding peptide from HBV. Nevertheless, the majority (~90%) of the peptide-reactive CTLs derived from this procedure failed to recognize a target cell line transfected and expressing the HBV antigen (Wentworth, *et al.*, *Molec. Immunol.* *32*:603 (1995)). To improve this procedure, we first studied the ability of DC to serve as APC for generating primary CTL responses using PBMC from normal (HBV negative) donors and the same HBV peptide previously studied. Because the numbers of circulating DC in peripheral blood are very low (< 1%), we generated DC in a 7-day culture of purified monocytes in the presence of

GM-CSF and IL-4 (Romani *et al.*, *Exp. Med. 180*:83 (1994); Sallusto, *et al.*, *J. Exp. Med. 179*:1109 (1994)).

PBMC from 12 HLA-A2.1 normal volunteers, all negative for HBV serological markers were used to generate DC and CTL responses to a known HBV CTL epitope. The *in vitro* generated DC were pulsed with the HBV peptide HBC_{18} (which binds with high affinity to HLA-A2.1), and subsequently used to induce CTL from autologous $CD8^+$ purified lymphocytes. The lymphocyte cultures were re-stimulated with antigen (peptide-pulsed autologous adherent monocytes) on days 7 and 14, and cytolytic activity was measured on day 21. The CTL induction cultures in each experiment (and for each blood donor) were maintained in separate microcultures (in 48 well-plates) throughout the entire procedure. Cytotoxic activity was measured in a 4-6 hr ^{51}Cr release assay using .221 (A2.1) cells that were either pulsed with peptide or not, and with a .221 (A2.1) cell line transfected with the HBV core gene. The results presented in Table I show that in all experiments performed with the 12 individuals, at least one culture had CTL that recognized peptide-pulsed target cells.

Table I. **Summary of results obtained in the induction of CTL using peptide-pulsed DC and IL-7 to a known HBV epitope in normal individuals**

Blood Donor[a]	No. of Cultures with Peptide-Reactive CTL[b]	No. of Peptide-Reactive CTL Recognizing .221 (A2.1).HB[c]
1	4/34(12)[d]	3/4(75)
2	3/48(6)	2/3(66)
3	4/48(8)	3/4(75)
4	1/48(2)	1/1(100)
5	3/48(8)	2/3(66)
6*	7/48(15)	7/7(100)
7	10/48(21)	10/10(100)
8	4/48(8)	4/4(100)
9	2/24(8)	2/2(100)
10	1/48(2)	1/1(100)
11	1/24(1)	1/1(100)
12	4/48(8)	4/4(100)

[a] All blood donors were HLA-A2+ and negative for HBV serum markers.
*Results obtained with donor No. 6 are presented in Table II.

[b] Cultures were scored as positive for CTL when % specific lysis in the presence of peptide was >20 over the value obtained in the absence of peptide.

[c] Cultures were scored as positive for killing the HBc transfected target cell line when the % specific lysis was >20 over the untransfected control.

[d] Numbers in parentheses represent percentages.

More important, most of the resulting peptide-reactive CTL (66 to 100%) were also capable of recognizing the .221(A2.1).HBC target cells which express, process and naturally present the HBC_{18} CTL epitope. An example of one of these experiments, presented in Table II, shows the cytotoxic activity of 14 of the 48 microcultures that were

set up for that specific experiment. The results show that 7 of these microcultures (marked with "**" in Table II) displayed high specific lytic activity against both peptide-pulsed and the HBV core-transfected target cells, .221 (A2.1).HBC.

Table II. Example of results obtained with peptide-pulsed DC in the induction of CTL to HBc_{18}

Culture No.[a]	% Specific Lysis[d]			GM-CSF[b] Before Ag	GM-CSF[b] After Ag	GM-CSF[c] Ratio
	.221(A2.1) + Pep	.221(A2.1) - Pep	.221(A2.1).HBc			
1	7	6	6	43.8	51.3	1.17
4*	4	3	6	36.6	223.9	6.12
9**	81	5	38	77.0	816.6	10.61
18**	27	1	13	57.5	637.0	11.07
22**	89	6	62	180.7	921.3	5.10
25**	92	2	23	120.9	912.7	7.55
26	3	1	2	59.1	48.3	0.82
28*	1	0	3	156.9	637.0	4.06
30**	59	3	25	111.8	582.0	5.21
33**	96	13	41	88.8	935.7	10.53
37*	6	5	5	162.8	558.8	3.43
40**	95	3	32	108.2	887.0	8.20
46	22	20	14	82.0	176.7	2.15
48	6	3	4	124.6	88.8	0.71

[a] Results from 14 of a total of 48 cultures have been selected as examples. Cultures marked with an "*" produced GM-CSF (>3 fold) as a result of Ag restimulation but had no cytolytic activity. Cultures marked with "**" produced GM-CSF and had significant specific lytic activity against both peptide-pulsed and transfected targets.

[b] GM-CSF concentrations in culture supernatants were determined prior Ag restimulation and 24 hr after Ag restimulation using an ELISA specific for human GM-CSF. Results are expressed as pg/ml.

[c] GM-CSF Ratio = GM-CSF produced after Ag restimulation/GM-CSF produced before Ag restimulation.

[d] Cytotoxic activity was determined in a 5-hr ^{51}Cr release assay using .221(A2.1) cells with and w/o peptide and .221(A2.1) cells transfected with the HBV core gene [.221(A2.1).HBc]. Effector: Target ratios were between 10:1 and 1:1.

In order to predict which microcultures may have antigen-specific CTL, cell culture supernatants from each well were removed just prior to, and 2 days after, the

second antigen-restimulation, and they were analyzed for the presence of GM-CSF. This cytokine can be produced by class I restricted CTL as a result of antigen challenge. The results, also presented in Table II, indicate that production of GM-CSF by the lymphocyte cultures induced by peptide re-stimulation and the cytolytic activity that was measured a week later correlated to a great extent. All seven cultures that had cytotoxic activity increased the production of GM-CSF at least 5 fold as a result of antigen restimulation. However, some cultures (marked with an "*") produced GM-CSF during the restimulation procedure but had no specific cytotoxic activity. Out of the 48 cultures there were only 4 (3 of them are in Table II) that displayed this pattern of reactivity. Thus, it is possible to predict with some degree of accuracy which cultures will result with antigen-specific CTL by their capacity to produce GM-CSF as a consequence of reacting with antigen.

Effects of the addition of lymphokines in the induction of CTL by peptide-pulsed DC

IL-7 enhances the *in vitro* generation of CTLs and promotes their growth (Celis, *et al.*, *Proc. Natl. Acad. Sci. (USA)* 91:2105 (1994)); Wentworth, *et al.*, *Molec. Immunol. 32*:603 (1995); Alderson, *et al.*, *J. Exp. Med. 173*:923)). Other lymphokines such as IL-12 and IL-10 have also been reported to enhance the production of CTL responses and to function as growth factor for CTLs (Chen, *et al.*, *J. Immunol. 147*:528 (1991); Yang, *et al.*, *J. Immunol. 155*:3897 (1995)). In order to assess whether IL-7, IL-10 and IL-12 could further enhance the induction of CTL by peptide-pulsed DC, we selected an experimental model system where, in our experience, suboptimal CTL responses were generally obtained.

CTL induction to melanoma-associated peptides using SAC-1-generated APC or DC is in general more difficult to achieve than CTL responses to the HBC_{18}, peptide (V. Tsai, unpublished). This could be due to a lower precursor frequency for CTL specific for melanoma-associated antigens, which in most cases represent tissue-specific "self" antigens that may induce some level of immunological tolerance. First, the effect of IL-10 was studied using DC pulsed with an HLA-A1-binding peptide from the melanoma-associated antigen *MAGE-3*. The results presented in Table III clearly demonstrate that IL-10 increased significantly the number of microcultures bearing antigen-specific CTL for both peptide-pulsed target cells and melanoma cells. While in

the absence of IL-10 only 1 out of 48 microcultures had CTL, the addition of IL-10 increased 10 fold the number of cultures yielding *MAGE-3* specific CTL.

Table III. Enhancement in the induction of tumor-specific CTL by IL-10 when using peptide-pulsed DC as antigen.

DC w/o IL-10[a]			DC + IL-10[a]		
Culture No.[b]	% Specific Lysis[c] (Δ Peptide)	% Specific Lysis[d] (Δ Tumor Cells)	Culture No.	% Specific Lysis[c] (Δ Peptide)	% Specific Lysis[d] (Δ Tumor Cells)
1	-1	0	1	3	-4
2	-1	-1	2	0	2
3	0	-2	3	16	9
4	-1	1	4	-1	1
5	0	5	5	1	-4
6	-1	-2	6	1	-1
7	-2	-19	7	23	6
8	1	0	8	53	-3
9	-2	1	9	7	2
10	-1	1	10	2	3
11	-2	0	11	2	8
12	3	7	12*	92	70
13	-1	1	13	1	-5
14	-1	0	14*	51	23
15	0	-5	15*	91	53
16	0	-6	16	1	-4
17	2	5	17	0	-4
18	-1	10	18*	56	55
19	0	0	19	1	12
20	-1	0	20	5	6
21	-3	1	21	1	5
22	-3	2	22	1	-14
23	-1	-4	23	15	11
24	0	0	24*	84	23
25	-2	1	25	-1	-1
26*	49	42	26	-1	-9

DC w/o IL-10[a]			DC + IL-10[a]		
Culture No.[b]	% Specific Lysis[c] (Δ Peptide)	% Specific Lysis[d] (Δ Tumor Cells)	Culture No.	% Specific Lysis[c] (Δ Peptide)	% Specific Lysis[d] (Δ Tumor Cells)
27	-1	2	27	1	1
28	-4	-9	28*	87	54
29	-2	3	29*	94	68
30	-3	1	30	0	0
31	-2	-1	31	4	0
32	1	0	32	-1	-27
33	-2	-2	33	-2	-3
34	-3	3	34*	73	15
35	-3	-1	35	3	-2
36	-4	-2	36	3	-1
37	-2	1	37*	83	52
38	-12	2	38	1	0
39	-3	2	39	0	-5
40	-2	3	40	3	-4
41	13	3	41	-1	-2
42	12	8	42*	84	64
43	-1	-6	43	12	3
44	19	5	44	3	4
45	-1	2	45	5	0
46	-1	-6	46	0	-2
47	-1	1	47	0	-1
48	-3	0	48	0	-8

[a] DC were prepared from PBMC as described in Materials and Methods and pulsed with the *MAGE-3*$_{161}$ peptide. IL-10 (10 ng/ml) was added on day 1 of the CTL induction cultures, and during the antigen re-stimulation procedure.

[b] Cultures marked with an "*" are considered to have specific CTL activity against both peptide-sensitized targets and melanoma cells.

[c] Specific lysis against the *MAGE-3*$_{161}$ peptide was measured using the Steinlin (HLA-A1$^+$) cells with and w/o peptide. Results are expressed as the difference (Δ) in the % lysis + peptide minus the % lysis w/o peptide.

[d] Specific lysis against melanoma cells was measured using the 938mel *(MAGE-3 $^+$)* and 888mel *(MAGE-3 $^-$)* cell lines. Results are expressed as the difference (Δ) in the lysis of 938mel minus the lysis of 888mel.

Next, the effect of various doses of IL-10 and the requirement for IL-7 were evaluated in two blood donors using an HLA-A2.1-binding peptide from the melanoma-associated antigen gp100. The results shown in Table IV make the following important points in regards to the role of these cytokines in the CTL induction protocol using DC as APC: a) in the absence of both IL-7 and IL-10 no CTL responses were observed; b) IL-10 alone, was not effective in inducing CTL to gp100; c) some CTL responses were observed in one of the donors when IL-7 was used alone, without IL-10; d) the use of both IL-7 and IL-10 in combination increased the frequency of microcultures displaying antigen-specific CTL in both blood donors; and e) the optimal dose of IL-10 in this system is approximately 10 ng/ml.

Table IV. Requirements for both IL-7 and IL-10 in the induction of CTL by peptide-loaded DC using a melanoma epitope from gp100

		Percent of positive cultures with CLT to gp100 $G9_{280}$[a]											
		"High" responding blood donor						Low" responding blood donor					
Target Cells:	IL-10(ng/ml)	0	10	0	10	30	100	0	10	0	10	30	100
	IL-7(ng/ml)	0	0	10	10	10	10	0	0	10	10	10	10
Peptide pulsed[b]		0	0	13	38	40	48	0	0	0	6	4	4
Melanoma[c]		NT[d]	NT	2	17	17	15	NT	NT	NT	2	4	4

[a] Numbers represent the % of wells, from a total of 48 that were set up for each condition.

[b] Cultures were scored as positive for peptide-reactive CTL if the difference in the % specific ^{51}Cr release obtained with targets loaded with peptide minus the value obtained with targets w/o peptide was 20% or more.

[c] Cultures were scored as positive for melanoma-reactive CTL if the difference in the % specific ^{51}Cr release obtained with $gp100^+$ melanoma cells minus the value obtained with melanoma gp100 cells was 20% or more.

Lastly, we wished to determine whether IL-12, another cytokine reported to enhance T cell activation and proliferation (Mehrotra, *et al.*, *J. Immunol. 151*:2444 (1993); Mehrotra, *et al.*, *J. Immunol. 154*:5093 (1995); Gately, *et al.*, *Cell Immunol. 143*:127 (1992); Chouaib, *et al.*, *Proc. Natl. Acad. Sci. (USA)* 91:12659 (1994); Fallarino, *et al.*, *J. Immunol. 156*:1095 (1996)), would further increase the effects of peptide pulsed DC, IL-7 and IL-10 in eliciting primary CTL responses *in vitro*. In three different experiments, IL-12 not only significantly increased the frequency of cultures

displaying specific CTL activity, but in some instances this lymphokine inhibited the CTL response. An example of one of these experiments, using "freshly-isolated" DC is presented in Table V.

Table V. Example: IL-12 Inhibits the generation of antigen-specific CTL by peptide pulsed "fresh" DC

DC w/o IL-12[a]			DC + IL-12		
Culture No.[b]	% Specific Lysis[c] (Δ Peptide)	% Specific Lysis[d] (Δ Tumor Cells)	Culture No.	% Specific Lysis[c] (Δ Peptide)	% Specific Lysis[d] (Δ Tumor Cells)
1	-10	-9	1	-1	3
2	0	-13	2	-2	-1
3*	64	87	3	2	5
4	12	1	4	-3	8
5	-3	-12	5	0	0
6*	24	38	6	1	1
7*	26	22	7	3	1
8	5	-1	8	-1	1
9	0	-5	9	0	4
10*	73	57	10	-2	-1
11	1	-5	11	0	-2
12	41	4	12	-2	29
13	3	-6	13	3	3
14*	73	72	14	3	1
15*	35	17	15	3	1
16*	84	76	16	2	-1
17	4	-8	17	3	6
18*	22	19	18	3	2
19	3	5	19	3	1
20*	85	76	20	3	3
21*	70	64	21	5	6
22	5	3	22	3	-1
23*	90	62	23	7	5
24	10	4	24	3	2
25	1	-2	25	-1	4

DC w/o IL-12[a]			DC + IL-12		
Culture No.[b]	% Specific Lysis[c] (Δ Peptide)	% Specific Lysis[d] (Δ Tumor Cells)	Culture No.	% Specific Lysis[c] (Δ Peptide)	% Specific Lysis[d] (Δ Tumor Cells)
26*	59	49	26	1	7
27*	77	77	27	-4	2
28	0	-4	28	-3	1
29*	70	49	29	-1	-1
30	3	8	30	1	1
31*	32	37	31	-1	-3
32	16	-4	32	-3	-4
33	5	-6	33	-2	0
34*	83	81	34	-2	-1
35	37	4	35	-1	-1
36	0	5	36	-1	1
37	0	-9	37	1	0
38*	17	18	38	-1	0
39	20	2	39	1	-1
40	0	-9	40	-1	-1
41*	45	32	41	4	1
42*	99	80	42	1	0
43	3	4	43	3	2
44	66	11	44	2	1
45*	87	17	45	2	2
46	8	-11	46	2	1
47	3	4	47	1	3
48	5	6	48	-1	3

a) "Fresh" DC were prepared from PBMC as described in Materials and Methods and pulsed with the gp100 peptide $G9_{280}$. IL-12 (10 ng/ml) was added on day 0 of the CTL induction cultures, and not used during the Ag re-stimulation procedures.

b) Cultures marked with an "*" are considered to have specific CTL activity against both peptide-sensitized targets and melanoma cells.

(c) Specific lysis against the $G9_{280}$ peptide was measured using the .221(A2.1) cells with and w/o peptide. Results are expressed as the difference (Δ) in the % lysis + peptide, minus the % lysis w/o peptide.

d) Specific lysis against melanoma cells was measured using the 624mel ($gp100^+$) and A375mel (gp^{100-}) cell lines. Results are expressed as the difference (Δ) in the lysis of 624mel minus the lysis of A375mel.

Expansion of peptide-induced CTL in tissue culture

One of the applications of the methods described here is in the area of adoptive therapy using antigen-specific CTL. If the peptide-induced CTL can be expanded in tissue culture in a similar way as tumor-infiltrating lymphocytes (TILs) and T cell clones, and maintain their specificity for antigen, these cells could be used for treating patients with on-going disease.

For these studies we evaluated the use of either a T-cell mitogen (anti-CD3 antibodies) or antigen (specific peptide) for their ability to activate and expand CTL that were induced with peptide-pulsed DC. The results presented in Figure 1 show that a MAGE-3 specific CTL line could be efficiently activated and expanded using either one of the T-cell activators. Although anti-CD3 antibodies were more efficient in promoting cell expansion than peptide-pulsed PBMC (1,300 fold versus 820 fold expansion in 13 days), cell cultures using peptide displayed higher cytolytic activity than the CTL grown in the presence of mitogen. These results were not totally unexpected since it is very likely that the T cell lines resulting from these cultures are not monoclonal and thus T cells for other specificities will be activated and expanded by the mitogen. In agreement with this, we have observed in many instances that repetitively stimulation of T-cell lines with mitogen results in total loss of cytotoxic activity (data not shown). This problem can be overcome by cloning the T-cells by limiting dilution (data not shown).

The capacity of anti-CD3 antibodies to expand CTL was also demonstrated with several gp100-specific CTL lines. The results presented in Table VI demonstrate that a 600 to 1500 fold expansion over a 15 day time period could be achieved and that high specific lytic activity was retained.

Table VI. Expansion efficiency of gp100-specific CTL lines using anti-CD3 monoclonal antibody and IL-2*

	Fold cell expansion at day:			% Specific Cytotoxicity (E:T, 20:1)			
CTL Line	11	13	15	.221(A2.1) + Pep ($FG9_{280}$)	.221(A2.1) - Pep	624mel ($gp100^+$)	A375mel ($GP100^-$)
3	250	513	1233	80	3	92	9
6	666	833	1500	100	5	94	38
30	208	455	1100	97	6	66	2
34	292	373	666	95	11	67	5
Average	*357*	*547*	*1129*	*93*	*6*	*80*	*14*

Four randomly selected CTL lines from the experiment described in Table IV were expanded using anti-CD3 antibodies, IL-2 and feeder cells as described in Materials and Methods.

EXAMPLE 4

Methods for identifying CTL-stimulating subdominant and immunodominant epitopes

Role of MHC Binding Affinity in Immunogenicity and Immunodominance

Previous studies had shown that HLA-A2-restricted CTL lines derived from melanoma patients recognize various peptides derived from the gp100 melanocyte-derived protein. Specifically, gp100-reactive CTL lines derived from tumor infiltrating lymphocytes (TIL) of 4 patients were screened for reactivity with a large panel of peptides containing HLA-A2.1 binding motifs (Kawakami, *et al.*, J. Immunol. 154:3961 (1995)). Only 7 of the 169 peptides tested were recognized by at least one of the CTL lines. Interestingly, the $G9_{280}$ was also reported by others as recognized by CTL from five melanoma patients (Cox, *et al.*, Science 264:716 (1994)).

In this example, nine-mer and ten-mer peptides corresponding to HLA-A2 binding motifs contained within the sequence of the known TAA gp100 were synthesized and tested for binding to purified HLA-A2.1 molecules. A total of 17 of the 136 (12.5 %) peptides tested had significant binding (IC_{50} < 500 nM) to HLA A2.1 molecules *in vitro* (Table VII).

Table VII. List of HLA-A2.1 Binding Peptides from the melanoma-associated antigen gp100

Peptide	Sequence[a]	A*0201 Binding[b] IC_{50} (nM)
$G9_{154}$	KTWGQYWQV	11.1
$G10_{476}$	VLYRYGSFSV	13.2
$G9_{178}$	MLGTHTMEV	14.3
$G10_{605}$	VLMAVVLASL	35.7
$G10_{224}$	ALDGGNKHFL	41.7
$G9_{619}$	RLMKQDFSV	45.5
$G10_{177}$	AMLGTHTMEV	52.1
$G10_{570}$	SLADTNSLAV	79.4
$G9_{209}$	ITDQVPFSV	83.3
$G9_{280}$	YLEPGPVTA	94.3
$G9_{11}$	HLAVIGALL	100.0
$G9_{606}$	LMAVVLASL	111.1
$G10_{57}$	RLDCWRGGQV	250.0
$G9_{18}$	LLAVGATKV	333.3
$G9_{592}$	GLGQVPLIV	500.0
$G10_{457}$	LLDGTATLRL	500.0
$G10_{544}$	VLPSPACQLV	500.0

[a] Underlined sequences represent CTL epitopes found on melanoma patients (Kawakami, *et al.*, *J. Immunol. 154*:3961 (1995); Bakker, *et al.*, *Int J Cancer 62*:97 (1995); Cox, *et al.*. *Science 264*:716 (1994).

[b] Measured as concentration (nM) of test peptide required for 50% inhibition of binding of standard peptide. See Materials and Methods for details.

Five of the known A2-restricted gp100 derived peptides are indeed contained within this set and the corresponding sequences are underlined in Table VII. With regards to the remaining two previously identified epitopes ($G10_{154}$ and $G10_{208}$), they displayed rather low MHC binding affinities (1010 nM and 2080 nM respectively, not included in Table VII). It should however be noted that these two 10-mers contain the two 9-mers $G9_{208}$ and $G9_{154}$, which have much higher binding affinity than the corresponding 10-mers and could indeed represent the real minimal epitopes.

From the results presented in Table VII, most significant is the observation that besides the five known A2-restricted dominant epitopes, at least twelve other peptides exist, which bind HLA-A2.1 molecules with equivalent affinities. The next series of experiments was designed to define the immunologic potential of these peptides.

In Vitro CTL Induction in Normal Individuals to gp100 Using Peptides Corresponding to Immunodominant Epitopes

We have reported that it is possible to elicit melanoma-reactive CTL using lymphocytes from normal individuals and MHC binding peptides corresponding to melanoma TAA (Celis, *et al.*, *Proc. Natl. Acad. Sci. (USA) 91*:2105 (1994)). Similar observations have also been reported by other groups (Houbiers, *et al.*, *Eur. J. Immunol. 23*:2072 (1993); Van, *et al.*, *Eur. J. Immunol. 24*:3038 (1994); Gagliardi, *et al.*, *Int. Immunol. 7*:1741 (1995); Bakker, *et al.*, *Cancer Res. 55*:5330 (1995); Plebanski, *et al.*, *Eur. J. Immunol. 25*:1783 (1994)).

However, technical difficulties have limited the usefulness of such protocols for the identification of CTL epitopes in humans. Specifically, it is commonly observed that these protocols result in the induction of low avidity CTL, and consequent lack of demonstrable recognition of cells that naturally process these epitopes (Wentworth, *et al.*, *Molec. Immunol. 32*:603 (1995)).

An improved *in vitro* CTL sensitization method was designed to solve these difficulties. The main features of this protocol are the use of dendritic cells (Bakker, *et al.*, *Cancer Res. 55*:5330 (1995); Young, *et al.*, *J. Exp. Med. 171*:1315 (1990); Mehta-Damani, *et al.*, *J. Immunol. 153*:996 (1994); Nair, *et al.*, *J. Virol. 67*:4062 (1993)) as a source of peptide-pulsed APC in combination with IL-7 (Kos, *et al.*, *Eur. J. Immunol. 22*:3183 (1992)) in the priming step and the addition of IL-10 (Chen, *et al.*, *J.*

Immunol. 147:528 (1991); Yang, *et al., J. Immunol 155*:3897 (1995)) during the antigen-restimulation cycles. This protocol is significantly more effective than previous protocols in eliciting high avidity CTL to known epitopes (E. Celis, *et al.*, in preparation).

Utilizing this method, five previously reported dominant gp100 peptides, underlined in Table VII, were tested for their capacity to induce CTL in at least two normal HLA-A2.1 blood donors. The results in Table VIII illustrate the type of CTL responses obtained in single-well cultures after 3 weekly cycles of antigen stimulation.

Table VIII. Examples of CTL responses to known epitopes from gp100 observed in normal individuals

		% Specific Cytotoxicity[a]			
Peptide	Sequence	.221(A2.1) + Peptide	.221(A2.1) - Peptide	624mel (gp100+)	A375mel (gp100-)
$G9_{154}$	KTWGQYWQV	61	1	39	6
		80	1	74	9
		78	1	3	10
$G10_{476}$	VLYRYGSFSV	82	1	30	1
		82	2	27	3
		97	1	3	2
$G9_{209}$	ITDQVDPFSV	77	7	790	14
		100	3	49	5
		81	3	3	3
$G9_{280}$	YLEPGPVTA	100	3	89	3[b]
		95	5	84	5
		33	9	2	5
$G10_{457}$	LLDGTATLRL	25	4	1	2

[a] The cytolytic activity of individual cultures (from a 48-well plate) was measured in a 5 hr ^{51}Cr release against four targets: .221(A2.1) plus peptide, .221(A2.1) w/o peptide, 624mel (A2+, grp100+) and A375 mel (A2+, gp100-). Effectors:target ratios were between 1:1 and 10:1. Numbers represent % specific cytotoxicity as defined in "Materials and Methods." Results in each row represent examples of individual wells from the same experiment performed with a specific peptide.

[b] This CTL line was restimulated with antigen and expanded for further characterization (Fig. 2).

A summary of the overall results from these experiments is also presented in Table IX.

Table IX. Summary of *in vitro* CTL responses from normal individuals to known epitopes

Peptide	Sequence	% Melanoma-Reactive CTL[a,c]	% Peptide-Reactive CTL[b,c]	Number of Cultures[d]	Number of Donors
$G9_{154}$	KTWGQYWQV	38	17	48	1
$G10_{476}$	VLYRYGSFSV	10	44	906	2
$G9_{209}$	ITDQVPSFSV	12	14	96	2
$G9_{280}$	YLEPGPVTA	13	9	216	5
$G10_{457}$	LLDGTATLRL	0	2	60	2

[a] These CTL killed both peptide-pulsed and melanoma (gp100+) targets.

[b] These CTL only killed peptide-pulsed targets.

[c] Percentages represent the number of positive wells/total X 100.

[d] Each culture represents an individual well of a 48-well tissue culture plate.

Taken together the results indicate that 4 of the 5 peptides tested were capable of inducing melanoma reactive CTL in approximately 10% of all cultures. The 4 peptides that induced CTL responses to gp100$^+$ melanoma cells were $G9_{154}$, $G10_{476}$, $G9_{209}$, and $G9_{280}$. Only the lowest affinity HLA-A2.1-binding peptide, $G10_{457}$, failed to elicit this kind of CTL. The results also show that all 5 peptides elicited at least some CTL specificities which recognized peptide-sensitized target cells but did not lyse melanoma cells.

Fine Antigen Specificity of $G9_{280}$ CTL Responses

One of the antigen specificity of CTL lines elicited with peptide $G9_{280}$ (marked with a footnote in Table VII) was further studied by testing its capacity to recognize various HLA-A2.1 melanoma cell lines and by determining the ability of peptide-pulsed, non-radiolabeled (cold) targets to inhibit the lysis of a gp100$^+$ melanoma cell line. The results presented in Figure 2A show that the $G9_{280}$ reactive CTL were capable of killing several HLA-A2.1 melanoma cell lines expressing gp100, but not a melanoma cell line that is negative for gp100. Furthermore, this reactivity against melanoma cells was specific since it was almost completely blocked by cold targets

loaded with the $G9_{280}$ peptide but not by cold targets pulsed with an irrelevant HLA-A2.1-binding peptide (Figure 2B).

Identification of gp100 Specific Subdominant CTL Epitopes.

As shown in Table VII, several gp100-derived peptides bind purified HLA-A2.1 molecules *in vitro*, and yet have not been reported in the literature as CTL epitopes recognized by melanoma patients. Specifically, although all of the peptides in Table VII were tested for reactivity with melanoma TIL from several patients, only 5 of the A2.1-binding peptides were demonstrated to function as dominant CTL epitopes. Next, six of the remaining gp100 derived peptides were tested using the *in vitro* priming protocol. The results in Table X demonstrate that 3 out of 6 peptides tested elicited melanoma-reactive CTL, and that 5 of these peptides could induce peptide-specific CTL which did not react with melanoma cells expressing gp100.

Table X. Examples of CTL responses to new epitopes from gp100 observed in normal individuals

		% Specific Cytotoxicity[a]			
Peptide[b]	Sequence	.221(A2.1) + Peptide	.221(A2.1) - Peptide	624mel (gp100+)	A375mel (gp100-)
$G9_{177}$	MLGTHTMEV	83	1	72	31[c]
		23	0	33	11
		16	1	15	23
$G10_{603}$	VLMAVVLASL	43	0	13	5
		73	4	6	19
		17	5	2	9
$G9_{619}$	RLMKQDFSV	14	9	3	2
		30	7	55	33
		15	5	4	26
$G9_{177}$	AMLGTHTMEV	98	0	83	14[c]
		54	0	65	2
		12	1	14	15
$G10_{270}$	SLADTNSLAV	81	1	29	18[c]
		42	0	25	4
		87	0	31	31

[a] The cytolytic activity of individual cultures (from a 48-well plate) was measured in a 5 hr^{51} Cr release against four targets: .221(A2.1) plus peptide, .221(A2.1) w/o peptide, 624mel (A2+, grp100+) and A375 mel (A2+, gp100-). Effectors:target ratios were between 1:1 and 10:1. Numbers represent % specific cytotoxicity as defined in "Materials and Methods." Results in each row represent examples of individual wells from the same experiment performed with a specific peptide.

[b] Results obtained with peptide $G10_{224}$ were all negative and are not presented in this table.

[c] These CTL lines were restimulated with antigen and expanded for further characterization (Figs. 3-5).

Table XI indicates that CTL responses to these peptides were somewhat more difficult to obtain than responses to the immunodominant epitopes recognized by melanoma patients (Table III), possibly indicating a less abundant TCR repertoire directed towards these peptide specificities.

Table XI. Summary of *in vitro* CTL responses from normal individuals to new gp 100 epitopes

Peptide	Sequence	% Melanoma-Reactive CTL[a,c]	% Peptide-Reactive CTL[b,c]	Number of Cultures[d]	Number of Donors
$G9_{178}$	MLGTHTMEV	3	2	60	2
$G10_{209}$	VLMAVVLASL	0	3	60	2
$G10_{224}$	ALDGGNKHFL	0	0	60	2
$G9_{639}$	RLMKQDFSV	0	7	60	2
$G10_{177}$	AMLGTHTMEV	10	2	60	2
$G10_{570}$	SLADTNSLAV	3	8	60	2

[a] These CTL killed both peptide-pulsed and melanoma (gp100+) targets.

[b] These CTL only killed peptide-pulsed targets.

[c] Percentages represent the number of positive wells/total X 100.

[d] Each culture represents an individual well of a 48-well tissue culture plate.

Antigen Specificity of CTL Elicited With HLA-A2.1-Binding Peptides

As shown in Table X, in many cases the levels of specific cytotoxicity of the peptide-induced CTL lines was not high, and in addition in many instances significant background lytic activity (killing of antigen-negative targets) was observed. In order to determine that the CTL responses elicited with these newly defined gp100 epitopes was indeed specific, some of the lymphocyte cultures (marked with a footnote in Table X) were further restimulated with antigen (peptide) and APC (for 2 additional cycles) and subsequently were tested for cytotoxicity at various effector:target rations. In addition, the ability to kill gp100 expressing melanoma cells was blocked using cold lymphoblastoid targets pulsed with synthetic peptides.

The results presented in Figure 3 show that CTL induced with $G9_{178}$ and $G10_{177}$ were highly specific for $gp100^+$ melanoma cells and that this activity could be inhibited by unlabeled (cold) target cells pulsed with the relevant gp100 peptide, but not by cold target cells pulsed with a control HLA-A2-high affinity binding peptide derived from the sequence of hepatitis B core protein, position 18-27.

The peptides $G9_{178}$ and $G10_{177}$ differ only by the presence of the alanine residue contained at the amino terminus of $G10_{177}$ (Tables VII and VIII). In order to determine whether these peptides represent a single or two separate epitopes, the CTL lines induced with $G9_{178}$ and $G10_{177}$ were tested against both peptides. The results presented in Figure 4 indicate that CTL induced with peptide $G10_{177}$ were unable to recognize peptide $G9_{178}$. On the other hand, as expected, CTL induced with $G9_{178}$ were capable of recognizing both peptides because the 10-mer peptide contained all residues present on the 9-mer. These results indicate that these two overlapping peptides are capable of eliciting two distinct CTL specificities and thus represent two separate epitopes.

The peptide $G10_{570}$ was also effective in eliciting anti-melanoma specific CTL. The data presented in Figure 5 illustrates that the $G10_{570}$ CFL line was capable of killing melanoma cell lines that express gp100 and that this lytic activity was inhibitable by cold targets loaded with $G10_{570}$, but not by cold targets sensitized with an irrelevant peptide.

In Vitro CTL Induction in Melanoma Patients to gp100 Using the $G10_{177}$ Epitope

The $G10_{177}$ epitope was tested for its ability to induce melanoma-reactive CTL using lymphocytes obtained from an HLA-A2.1 melanoma patient. Forty eight individual CTL cultures were established and after three rounds of antigen stimulation one culture gave forth to melanoma-reactive CTL. The results presented in Figure 6 show that the melanoma patient CTL induced with peptide $G10_{177}$ effectively and specifically recognized both peptide-sensitized targets and $gp100^+$ melanoma cells and that the lytic activity against melanoma cells was blocked by cold target cells pulsed with the relevant peptide.

The present invention led to the identification of at least 3 new gp100 epitopes capable of eliciting tumor-reactive CTLs. Since only 6 of 12 potential subdominant epitopes have been tested to date following our dendritic cell priming *in vitro* immunization protocol, it is likely that additional gp100 derived epitopes will also be identified. The experimental protocol described herein, allows to define the CTL epitopes contained within a given tumor antigen (regardless of whether they are immunodominant or not) and fully exploit the immunological potential of the tumor antigen itself.

In vivo and *in vitro* CTL inductions using both dominant and subdominant epitopes will yield a more vigorous and diverse immune response. Polyvalent vaccines containing mixtures of epitopes from several TAA will overcome the need for complex mixtures of whole proteins or genes. Immunization with subdominant epitopes will focus CTL responses toward specific conserved sequences and thus prevent the emergence of tumor variants during the course of disease.

Furthermore, the results presented here also demonstrate that it is possible to induce tumor-reactive CTL from blood of cancer patients specific for both dominant and subdominant epitopes. These CTL could be expanded in culture to approximately 1 X 10^9 cells (data not shown), still retained antigen specificity and could thus be used for adoptive therapies. Immune therapy using peptide-primed CTL from blood may be a convenient (since no tumor biopsy is required), and reliable (because of the high degree of specificity) alternative to the already promising current TIL based immunotherapies (Rosenberg, *et al.*, *J. Natl. Cancer Inst.* 86:1159 (1994)).

EXAMPLE 5

Expansion of CTLs Recognize MAGE Antigens.

MAGE2 and *MAGE3* antigens are not only frequently expressed in melanoma tumors, but also in approximately 40-60% of epithelial tumors (colon, breast, lung) (Weynants, P. *et al.*, *International Journal of Cancer* 56:826-829 (1994); De Plaen, E. *et al.*, *Immunogenetics* 40: 360-369 (1994); Russo, V. *et al.*, *International Journal of Cancer* 64:216-221 (1995)). HLA-A1 (Celis, E. *et al.*, Proceedings of the National Academy of Sciences of the United States of America 91:2105-2109 (1994); Gaugler, B. *et al.*, *J Exp Med.* 179:921-930 (1994)) and HLA-A2.1 (van der Bruggen, P. *et al.*, *Eur J Immunol.* 24: 3038-3043 (1994)) restricted *MAGE3*-derived CTL epitopes have been reported. Those studies demonstrated how CTL induced *in vitro* using the peptides EVDPIGHLY (in the case of HLA-A1) and FLWGPRALV (in the case of HLA-A2.1) recognized peptide-sensitized EBV-transformed cell lines and also killed *MAGE3*$^+$ melanoma cell lines (Celis, E. *et al.*, Proceedings of the National Academy of Sciences of the United States of America 91:2105-2109 (1994); Gaugler, B. *et al.*, *J Exp Med.* 179:921-930 (1994); van der Bruggen, P. *et al.*, *Eur J Immunol.* 24: 3038-3043 (1994)).

In order, to identify additional HLA-A2.1-restricted CTL epitopes derived from the *MAGE2* and *MAGE3* antigens, a total of 81 and 89 synthetic peptides from *MAGE2* and *MAGE3* respectively, 9 to 11 residues long, all containing HLA-A2.1 binding motifs (Ruppert, J. *et al.*, *Cell* 74:929-937 (1993); Rammensee, H.-G. *et al.*, *Immunogenetics* 41:178-228 (1995)) were tested for their capacity to bind purified HLA-A2.1 molecules *in vitro*. Eight of 81 peptides from *MAGE2* and 9 of 89 peptides from *MAGE3* bound to HLA-A2.1 molecules with an $IC_{50} \leq 500$ nM (Table XII), which approximates the affinity threshold generally associated with CTL immunogenicity (Sette, A. *et al.*, *Journal of Immunology* 153:5586-5592 (1994)).

Table 12. *List of HLA-A2.1 binding peptides from MAGE-2 and MAGE-3*

Peptide[a]	Sequence	A*0201 Binding[b]	No. of cultures containing CTL reactive with: [c]	
		IC_{50} (nM)	Peptide	Tumor cells
MAGE2[11_{112}]	KMVELVHFLLL	5.0	5	0
MAGE2[9_{112}]	KMVELVHFL	9.8	1	0
MAGE2[10_{112}]	KMVELVHFLL	22.7	4	0
MAGE2[10_{157}]	YLQLVFGIEV	50.0	13	3[d]
MAGE2[9_{153}]	KASEYLQLV	151.5	1	0
MAGE2[9_{220}]	KIWEELSML	166.7	NT[e]	NT
MAGE2[9_{271}]	FLWGPRALI	238.1	NT	NT
MAGE2[10_{160}]	LVFGIEVVEV	357.1	NT	NT
MAGE3[11_{159}]	QLVFGIELMEV	7.9	3	0
MAGE3[11_{195}]	IMPKAGLLIIV	20.0	3	0
MAGE3[9_{271}]	FLWGPRALV[f]	31.3	15	1
MAGE3[10_{112}]	KVAELVHFLL	54.3	0	0
MAGE3[11_{174}]	HLFIYATCLGL	55.6	NT	NT
MAGE3[10_{44}]	TLVEVTLGEV	66.6	NT	NT
MAGE3[9_{112}]	KVAELVHFL	68.4	5	3[g]
MAGE3[10_{160}]	LVFGIELMEV	76.9	1	0
MAGE3[9_{174}]	YIFATCLGL	185.2	NT	NT

a Numbers in parentheses represent peptide size and the position on the protein sequence (subscript)

b Measured as concentration (nM) of test peptide required for 50% inhibition of binding of the standard. See *Materials and Methods* for details.

c Cultures were considered containing CTL when specific cytotoxicity towards antigen-containing target (plus peptide or MAGE+ tumor, 624mel) was > 15% above cytotoxicty with negative control target. Values represent the number of positive cultures out of 48 that were tested.

d These cultures had > 10% specific cytotoxicity towards tumor cells after 2 rounds of restimulation, however, the intensity of the anti-tumor response increased after T-cell cloning (Figs 1 and 2).

e Not tested

f This peptide was described as an HLA-A2 restricted epitope (41). Cytotoxicity to tumor cells was observed after 4 rounds of restimulation with peptide.

g CTL with this reactivity were obtained in 3 out of 4 different lymphocyte donors.

Several of the HLA-A2.1 binding peptides from *MAGE2* and *MAGE3* (Table XII) were tested for their capacity to induce CTL using a peptide-pulsed dendritic cell (DC) primary *in vitro* immunization protocol (Tsai, V. *et al.*, *Journal of Immunology* 158:1796-1802 (1997)). Most peptides (5 out of 5 from *MAGE2* and 5 out of 6 from *MAGE3*) induced CTL that killed peptide-sensitized (EBV-transformed) target cells (Table XII).

Selected CTL cultures were expanded by repeated antigen restimulations (peptide and APC) to obtain sufficient effector cells for more extensive cytotoxicity assays. Specifically, peptide-reactive CTL lines were studied for their ability to recognize tumor cells expressing the corresponding *MAGE* antigens. In the majority of cases, the reactivity towards peptide-sensitized target cells remained evident. Furthermore, MAGE2[10_{157}]-reactive CTL lines exhibited the capacity to kill melanoma tumor cells, which naturally produce and process the *MAGE2* protein. Similarly, at least 2 of the 6 HLA-A2.1 binding peptides from *MAGE3*, MAGE3[9_{271}] and MAGE3[9_{112}], were found to elicit melanoma-reactive CTL (Table XII).

To study in more detail the antigen specificity and tumor reactivity of the MAGE2[10_{157}] and MAGE3[9_{112}]-reactive CTL, T-cell clones were isolated from cultures which exhibited anti-melanoma reactivity. On the other hand, since MAGE3[9_{271}], had already been reported as an epitope for melanoma-reactive CTL (van der Bruggen, P. *et al.*, *Eur J Immunol.* 24: 3038-3043 (1994)), the corresponding CTL lines recognizing this epitope were not further characterized.

The results shown in Figure 7 demonstrate that the CTL induced by MAGE2[10_{157}]peptide effectively killed not only melanoma cells but also colon and gastric tumor cell lines, provided that they express both HLA-A2 and *MAGE2*. Furthermore, recognition of melanoma, colon cancer cells, and to some extent gastric cancer cells, could be blocked by unlabeled (cold) target cells pulsed with MAGE2[10_{157}], thus confirming the antigen specificity of this CTL clone (Figure 6).

The antigen-specificity and anti-tumor reactivity of an anti-MAGE3[9_{112}] CTL clone was also similarly determined. The results presented in Figures 9 and 10 demonstrate that this CTL clone was also highly specific and capable of recognizing HLA-A2 positive melanoma, colon and gastric tumor cells which express the *MAGE3* protein.

In summary, these results illustrate the identification of 2 novel HLA-A2.1-restricted *MAGE*-derived epitopes, capable of eliciting antigen specific CTL. These CTL recognized and lysed not only peptide-sensitized target cells, but also tumor cell lines of various histologic types.

EXAMPLE 6

Expansion of CTLs that recognize CEA Antigen.

CEA (carcinoembryonic antigen) is a TAA frequently expressed in epithelial tumors such as lung, colon, gastric and breast carcinomas (Vincent, R. G. *et al.*, *Cardiovasc. Surg.* 66: 320-328 (1978); Shavely, J. *et al.*, CRC Critical Reviews in Oncology and Hematology 2:355-399 (1985); Thompson, J., *et al.*, *Journal of Clinical and Laboratory Analytics* 5:344-366 (1991); Sikorska, H. *et al.*, *Cancer Detection and Prevention* 12:321-355 (1988); Muraro, R. *et al.*, *Cancer Research* 45: 5769-5780 (1985); Steward, A. M. *et al.*, *Cancer* 33:1246-1252 (1974)). Following the approach outlined in the previous section for *MAGE2* and *MAGE3*, the amino acid sequence of CEA was scanned for HLA-A2.1 binding motifs, also taking into account the effect of secondary anchor residues associated with high affinity MHC binding (Ruppert, J. *et al.*, *Cell* 74:929-937 (1993)). Of a total of 18 motif-containing peptides (9mers and 10mers) tested for HLA-A2.1 binding, 9 bound to HLA-A2.1 with an IC_{50} of 500 nM or less

Table 13. *List Of HLA-A2.1 binding peptides from CEA*

Peptide[a]	Sequence	A*0201 Binding[b] IC_{50}(nM)	No. of cultures containing CTL reactive with:[c] Peptide	Tumor cells
$CEA[10_{176}]$	YLWWVNNQSL	26.3	0	0
$CEA[9_{605}]$	YLSGANLNL[d]	27.8	9	1
$CEA[10_{532}]$	YLWWVNGQSL	33.3	NT[f]	NT
$CEA[10_{690}]$	GIMIGVLVGV	56.8	NT	NT
$CEA[9_{691}]$	IMIGVLVGV	68.5	10	1[e]
$CEA[9_{24}]$	LLTFWNPPT	178.6	0	0
$CEA[9_{589}]$	VLYGPDTPI	200.0	2	0
$CEA[10_{691}]$	IMIGVLVGVA	227.3	NT	NT
$CEA[10_{233}]$	VLYGPDAPTI	454.5	NT	NT

a Numbers in parentheses represent peptide size and the relative position in the protein sequence (subscript).

b Measured as concentration (nM) of test Peptide required for 50% inhibition of binding of standard peptide. See *Materials and Methods* for details.

c Cultures were considered containing CTL when specific cytotoxicity towards antigen-containing target (Plus Peptide or CEA+ tumor, SW403) was > 15% above cytotoxicity with negative control target. Values represent the number of positive cultures out of 48 that were tested.

d This peptide was described as an HLA-A2 restricted epitope (13). Although cytotoxicity 10 tumor cells was observed, specificity could not be demonstrated by cold target inhibition (not shown).

e Similar results were obtained from 2 (out of 3) different lymphocyte donors. Cytotoxicity to tumor cells was observed after 4 rounds of restimulation with peptide.

f Not tested.

Interestingly, the second highest binder, peptide CEA[9_{605}] was recently reported as a CTL epitope in cancer patients after vaccination with a recombinant CEA construct (Tsang, K. Y. *et al.*, *Journal of the National Cancer Institute*, 87:982-990 (1995)).

Several other HLA-2.1-binding peptides derived from CEA were studied for their capacity to elicit CTL using DC as antigen-presenting cells. Three of 5 peptides tested, stimulated CTL responses which recognized peptide-sensitized target cells. Moreover, after restimulation with antigen and APC, killing of CEA-expressing tumor cells was demonstrated for CEA[9_{605}] and CEA[9_{691}] specific CTL (Table XIII).

The CTL line reactive with peptide CEA[9_{691}] was cloned and studied further in terms of specificity and recognition of various CEA-expressing tumor cells. Cloned CTL specific for CEA[9_{691}] had high specific lytic activity towards tumor target cells (colon and gastric) that express HLA-A2 and CEA (Figure 11). Furthermore, the killing activity directed against gastric and colon tumor cells was specific since it was blocked with "cold targets" pulsed with peptide CEA[9_{691}] but not with targets pulsed with a control HLA-A2.1 binding peptide (Fig. 12).

EXAMPLE 7

Expansion of CTL That Recognize HER-2/*neu* Antigen.

Another TAA frequently found on epithelial tumors is the product of the HER-2/*neu* gene, also known as cerbB-2. This TAA is a cell surface protein often overexpressed by breast and ovarian carcinomas, and also expressed by colon and lung carcinomas (Slamon, D. J. *et al.*, *Science* 244:707-712 (1989); Yokota, J. *et al.*, *Lancet* 1:765-767 (1986)). Out of 165 peptides (9mers and 10mers) containing HLA-A2.1 binding motifs, 23 were found to bind with an $IC_{50} \leq 500$ nM to purified HLA-A2.1 molecules (Table XIV).

Table 14 *List of HLA-A2.1 binding peptides from HER-2/neu*

Peptide[a]	SEQUENCE	A*0201 Binding[b] IC_{50}(nM)	No. of cultures containing CTL reactive with:[c] Peptide	Tumor cells
HER2[9_{365}]	VVLGVVFGI	14.3		
HER2[9_{106}]	QLFEDNYAL	17.2		
HER2[9_{689}]	RLLQETELV	20.8		
HER2[9_{153}]	VLIQRNPQL	22.7		
HER2[9_{369}]	KIFGSLAFL[d]	33.3	29	2
HER2[9_{653}]	SIISAVVGI	69.4		
HER2[9_{435}]	ILHNGAYSL	74.6	2	2
HER2[10_{952}]	YMIMVKCWMI	83.3		
HER2[9_{5}]	ALCRWGLLL	100	7	1
HER2[10_{755}]	LLGICLTSTV	102		
HER2[10_{5}]	ALCRWGLLLA	138.9		
HER2[9_{789}]	CLTSTVQLV[d]	147.1	3	0
HER2[9_{48}]	HLYQGCQVV[e]	147.1	3	0
HER2[9_{790}]	QLMPYGCLL	217.4		
HER2[9_{952}]	YMIMVKCWM	217.4		
HER2[9_{466}]	ALIHHNTHL	238.1		
HER2[9_{797}]	ILDEAYVMA	238.1		
HER2[10_{434}]	RILHNGAYSL	277.8		
HER2[10_{773}]	VMAGVGSPYV[d]	277.8		
HER2[10_{144}]	SLTEILKGGV	333.3		
HER2[10_{106}]	QLFEDNYALA	357.1		
HER2[9_{308}]	GLACHQLCA	416.7		
HER2[10_{654}]	IISAVVGILL	416.7		

Table 14. *List of HLA-A2.1 binding peptides from HER-2/neu*

(Footnotes for Table 14.

a Numbers in parentheses represent peptide size and the relative position in the protein sequence (subscript).

b Measured as concentration (nM) of test peptide required for 50% inhibition of binding of standard peptide. See *Materials and Methods* for details.

c Cultures were considered containing CTL when specific cytotoxicity towards antigen-containing target (plus peptide or HER+ tumor, SW403) was > 15% above cytotoxicity with negative control target. Values represent the number of positive cultures out of 48 that were tested.

d These peptides have been described as HLA-A2 restricted epitopes (10, 11, 47).

Amongst the highest affinity HLA-A2.1 binding peptides, it was noted the HER2[9_{369}] peptide, which has been previously reported as a CTL epitope in cancer patients (Fisk, B. *et al.*, *J. Exp. Med.*, 181:2109-2117 (1995)).

To identify additional HER-2/*neu*-derived CTL epitopes, we selected 4 of the HLA-A2.1 binding peptides (HER2[9_{435}], HER2[9_{789}], HER2[9_{48}] and HER2[9_5]) and tested them using our *in vitro* immunization protocol. HER2[9_{369}] was included in this analysis to confirm that this epitope was capable of inducing tumor-reactive CTL in our system. All 5 HLA-A2.1 binding peptides tested elicited CTL that killed peptide-sensitized target cells. Three of them (HER2[9_{369}], HER2[9_{435}] and HER2[9_5] elicited CTL which recognized and specifically killed tumor cells expressing the HLA-A2.1 and HER-2/*neu* antigens (Table XIV). Since HER2[9_{369}] has been described in the past as an epitope for tumor-reactive CTL (Fisk, B. *et al.*, *J Exp Med*, 181:2109-2117 (1995)), we did not further characterize these CTL. By contrast, CTL lines specific for HER2[9_{435}] and HER2[9_5] were expanded for further analysis.

As shown in Figure 13 the CTL line induced with HER2[9_{435}] killed both peptide sensitized target cells and HLA-A2.1, HER-2/*neu* positive tumor cells (Fig. 13A). Furthermore, recognition of the tumor cell targets was again significantly inhibited by unlabeled (cold) targets pulsed with HER2[9_{435}], but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (Fig. 13B). These results

demonstrate that recognition of the tumor cells by these CTL is indeed antigen-specific. Similar results were obtained with the CTL line specific for HER2[9_5], similarly tested for tumor cell reactivity and antigen specificity (Figure 14).

EXAMPLE 8

Engineered CEA-Specific CTL Epitopes.

Previous studies have shown that it is possible to increase the antigenicity by modification of primary or secondary anchors (Parkhurst, M. R. *et al.*, *Journal of Immunology* 157: 2539-2548 (1996); Topalian, S. L. *et al.*, *Journal of Experimental Medicine* 183: 1965-1971 (1996)). As shown in Table XIII, CEA[9_{24}] missing one of the "canonical" A2.1 anchors (a V at the carboxyl terminal end), as defined by pool sequencing analysis (Rammensee, H.-G. *et al.*, *Immunogenetics* 41:178-228 (1995)). In order to determine whether substituting the non-canonical C-terminal anchor (T) with the canonical residue, 2 analogue peptides from CEA[9_{24}] were prepared and tested for binding to purified HLA-A2.1 molecules. These two analogs differed by the residue incorporated in position 2, specifically either the naturally occurring "L" (CEA[9_{24}]-L2V9, sequence: LLTFWNPPV) or an "M" residue (CEA[9_{24}]-M2V9, sequence: LMTFWNPPV), based on the finding that presence of "M" in position 2 is frequently associated with optimal HLA-A2.1 binding capacity (Ruppert, J. *et al.*, *Cell* 74:929-937 (1993); del Guercio, M. F. *et al.*, *Journal of Immunology* 154: 685-693 (1995)). The analogue peptides bound to purified HLA-A2.1 molecules with an approximately 10-fold increased affinity, in the case of CEA[9_{24}]-L2V9 (IC_{50} 16 nM), and 40-fold increase for CEA[9_{24}]-M2V9 (IC_{50} 4.5 nM), as compared to the natural sequence, CEA[9_{24}] (IC_{50} 178.6 nM).

It was notable that although peptide CEA[9_{24}] was not capable, at least according to our experimental protocol, of triggering CTL responses *in vitro* (Table XIII), both peptide analogs were immunogenic in terms of CTL induction. CTL lines induced by both analogs specifically recognized and killed SW403 colon cancer cells expressing the CEA and HLA-A2 antigens (data not shown). CEA[9_{24}]-M2V9-specific CTL were cloned and further characterized. The results presented in Figure 15 demonstrate that

these CTL were indeed antigen specific, and recognized HLA-A2$^+$ gastric and colon tumor cell lines which express CEA.

EXAMPLE 9

Crossreactive Binding of Tumor CTL Epitopes to HLA Supertype Molecules.

The results presented above illustrate the identification of multiple epitopes from various TAA. It is anticipated that the combined use of such epitopes could provide effective coverage of a majority of breast, ovarian, lung and colon carcinomas.

To examine their relative potential population coverage, we examined whether these epitopes could bind other common alleles of the A2 supertype (del Guercio, M. F. *et al.*, *Journal of Immunology* 154: 685-693 (1995); Sidney, J., Grey *et al.*, *Immunology Today* 17:261-266 (1996)). HLA-A2.2 and -A68.2 were selected because of their high frequency in the black population and -A2.3 and -A2.6 were chosen because of their high frequency in Oriental ethnicity (del Guercio, M. F. *et al.*, *Journal of Immunology* 154: 685-693 (1995); Sidney, J., Grey *et al.*, *Immunology Today* 17:261-266 (1996)).

In addition to binding to HLA-A2.1 most of the peptides representing CTL epitopes could also bind to at least another member of the HLA-A2 supertype (Table XV).

Table 15. *A2 supertype binding data from various CTL epitopes*

Peptide	Sequence	A*0201[a] IC_{50} (nM)	A*0202 IC_{50} (nM)	A*0203 IC_{50} (nM)	A*0206 IC_{50} (nM)	A*6802 IC_{50} (nM)	Supertype Binder[b]
MAGE2[10_{157}]	YLQLVFGIEV	50	165	345	370	9302	4
MAGE3[9_{271}]	FLWGPRALV	31.3	43	14	336	40	5
MAGE3[9_{112}]	KVAELVHFL	68.4	29	14	168	17	5
CEA[9_{605}]	YLSGANLNL	27.8	165	2.4	804	>10000	3
CEA[9_{691}]	IHIGVLVGV	68.5	62	13	106	89	5
CEA[9_{24}]	LLTFWNPPT	178.6	1720	67	755	>10000	2
CEA[9_{24}]-L2V9	LLTFWNPPV	16	297	26	56	9442	4
CEA[9_{24}]-M2V9	LMTFWNPPV	4.5	779	7.6	33	3448	3
HER2[9_{369}]	KIFGSLAFL	33.3	9.0	19	23	3333	4
HER2[9_{789}]	CLTSTVQLV	147.1	457	6.7	308	8000	4
HER2[10_{773}]	VMAGVGSPYV	277.8	391	13	3700	>10000	3
HER2[9_{435}]	ILHNGAYSL	74.6	358	100	569	>10000	3
HER2[9_5]	ALCRWGLLL	100	>10000	278	8863	>10000	2

a) Data exported from Tables 1, 2 and 3 (for comparison).

b) Represents number of alleles binding with $IC_{50} \leq 500$ nM out of the 5 tested.

Specifically, both of the epitopes from *MAGE3*, MAGE3[9_{271}] and MAGE3[9_{112}], bound well ($IC_{50} \leq 500$ nM) all five members of the A2 supertype studied. Similarly, peptide MAGE2[10_{157}] bound 3 of the 5 alleles of the A2 supertype (Table XV). In regards to the CTL epitopes from CEA, peptides CEA[9_{605}] and CEA[9_{691}] were found to bind to 4 and 5 of the A2 supertype alleles respectively (Table XV). Most significant was the observation that while CEA[9_{24}] could only bind to 2 of the 5 alleles, the CEA[9_{24}]M2V9 and CEA[9_{24}]L2V9 analogs, which were found in the present study to be immunogenic, bound 3 and 4 of the 5 A2 supertype alleles tested respectively (Table XV). Finally, peptides representing various CTL epitopes from HER-2/*neu* were found to bind to more than one of the five A2 supertype alleles (Table XV).

In summary, these results indicate that the majority of the HLA-A2.1-restricted CTL epitopes that were identified in the present and previous reports have the capacity to bind to several of the members of the A2 supertype. Studies are underway to determine whether the engineering approach can also further increase the crossreactive binding of peptides such as HER2[9_5] (sequence ALCRWGLLL), by substituting the primary and/or secondary binding anchors.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims. All references and patents cited herein are incorporated by reference for all purposes.

WHAT IS CLAIMED IS:

1. A method for producing antigen-specific cytotoxic T cells *in vitro* comprising:

contacting immunogenic peptides from an antigen that binds class I MHC molecules with antigen presenting cells pretreated with pretreatment growth factors; and

incubating the antigen presenting cells with purified CD8 cells in the presence of two or more incubation growth factors, thereby producing antigen-specific cytotoxic T cells.

2. The method of claim 1 wherein the antigen presenting cells are dendritic cells.

3. The method of claim 2, wherein the dendritic cells are prepared by incubating dendritic cell precursors in the presence of pre-treatment growth factors selected from the group consisting of GM-CSF and IL-4.

4. The method of claim 1, wherein desired immunogenic peptides are contacted with MHC molecules by incubating the antigen presenting cells with about 10 to 50 μg/ml immunogenic peptide.

5. The method of claim 1, wherein the immunogenic peptides are identified by selecting peptides from a pathogen or tumor-associated antigen that bear a desired MHC binding motif.

6. The method of claim 1 wherein one incubation growth factor is IL-7.

7. The method of claim 1 wherein one incubation growth factor is IL-10.

8. The method of claim 1 wherein the incubation growth factor IL-7 is added at the start of the incubation step and the incubation growth factor IL-10 is added one day later.

9. The method of claim 1, further comprising contacting the antigen specific cytotoxic T cells with a pharmaceutically acceptable carrier, thereby forming a pharmaceutical composition, and administering the pharmaceutical composition to a patient.

10. The method of claim 1 wherein the cytotoxic T cells are useful in the treatment of cancer, AIDS, hepatitis, bacterial infection, fungal infection, malaria or tuberculosis.

11. A method of specifically killing target cells in a human patient, comprising:

obtaining a fluid sample containing cytotoxic T cells from a first patient;

contacting the cytotoxic T cells with antigen presenting cells pretreated with pre-treatment growth factors, wherein the antigen presenting cells comprise class I MHC molecules;

incubating said pretreated antigen presenting cells with the cytotoxic T cells in the presence of desired immunogenic peptides and two or more incubation growth factors, thereby producing activated cytotoxic T cells;

contacting the activated cytotoxic T cells with an acceptable carrier, thereby forming a pharmaceutical composition; and

administering the pharmaceutical composition to a patient.

12. The method of claim 11 wherein the antigen presenting cell is a dendritic cell.

13. The method of claim 12, wherein the dendritic cells are prepared by incubating dendritic cell precursors in the presence of pre-treatment growth factors selected from the group consisting of GM-CSF and IL-4.

14. The method of claim 11, wherein desired immunogenic peptides are contacted with MHC molecules by incubating the antigen presenting cells with about 10 to 50 μg/ml immunogenic peptide.

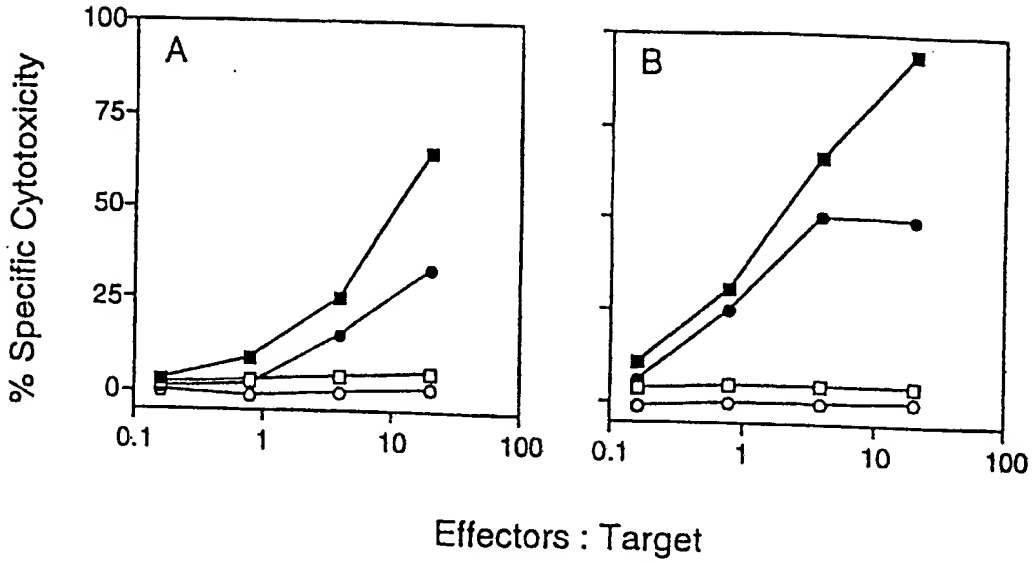
15. The method of claim 11, wherein the immunogenic peptides are selected by selecting peptides from a pathogen that bear a desired HLA-binding motif.

16. The method of claim 11 wherein one incubation growth factor is IL-7.

17. The method of claim 11 wherein one incubation growth factor is IL-10.

18. The method of claim 11 wherein the incubation growth factor IL-7 is added at the start of the incubation step and the incubation growth factor IL-10 is added one day later.

19. The method of claim 11 wherein the cytotoxic T cells are useful in the treatment of cancer, AIDS, hepatitis, bacterial infection, fungal infection, malaria or tuberculosis.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100
75
50
25
0
A
B
0.1
1
10
100
Effectors : Target

FIGURE 1

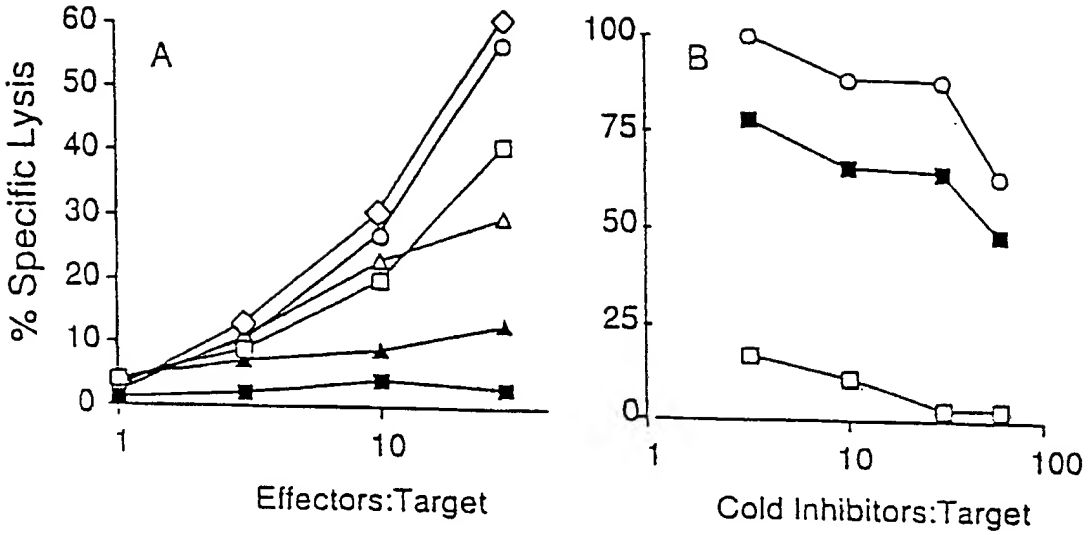

A
% Specific Lysis
60
50
40
30
20
10
0
1
10
Effectors:Target
B
100
75
50
25
0
1
10
100
Cold Inhibitors:Target

FIG. 2A

FIG 2B

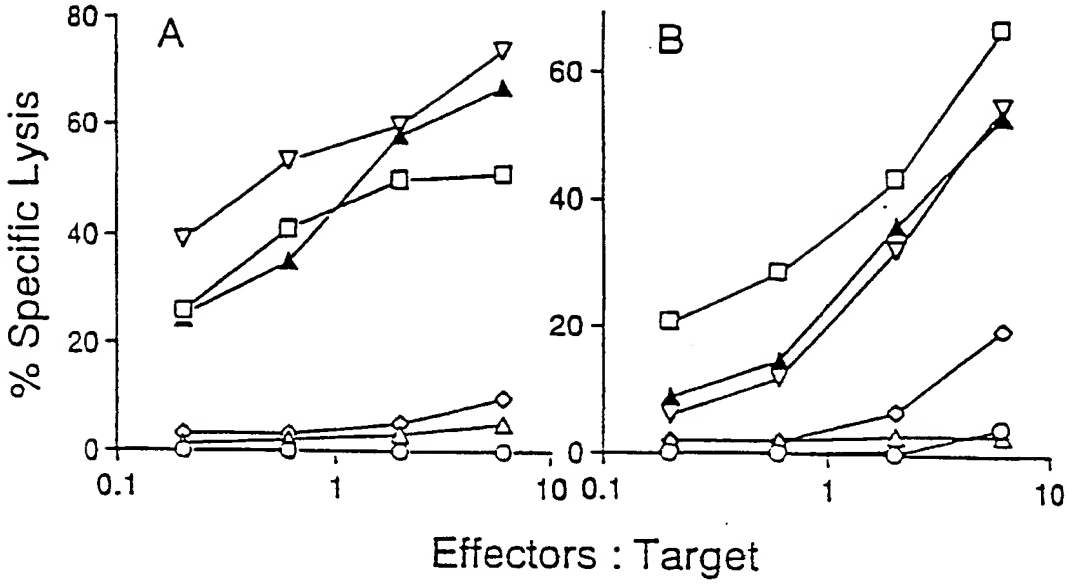
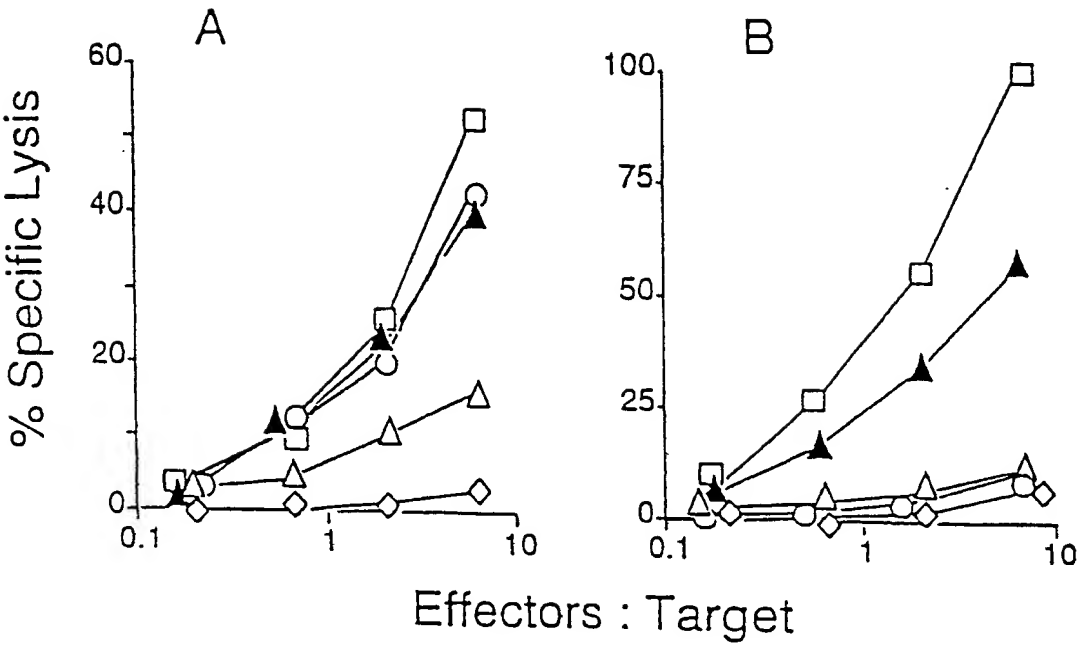


FIG. 3A

FIG. 3B


A
B
% Specific Lysis
60
40
20
0
100
75
50
25
0
0.1
1
10
0.1
1
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Effectors : Target

FIG. 4A

FIG. 4B

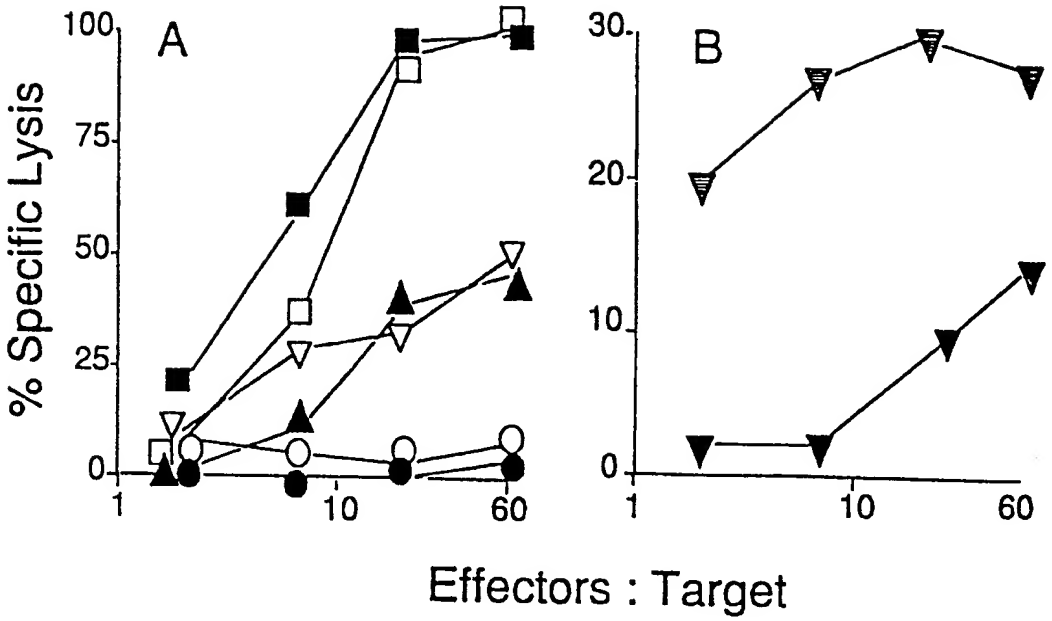
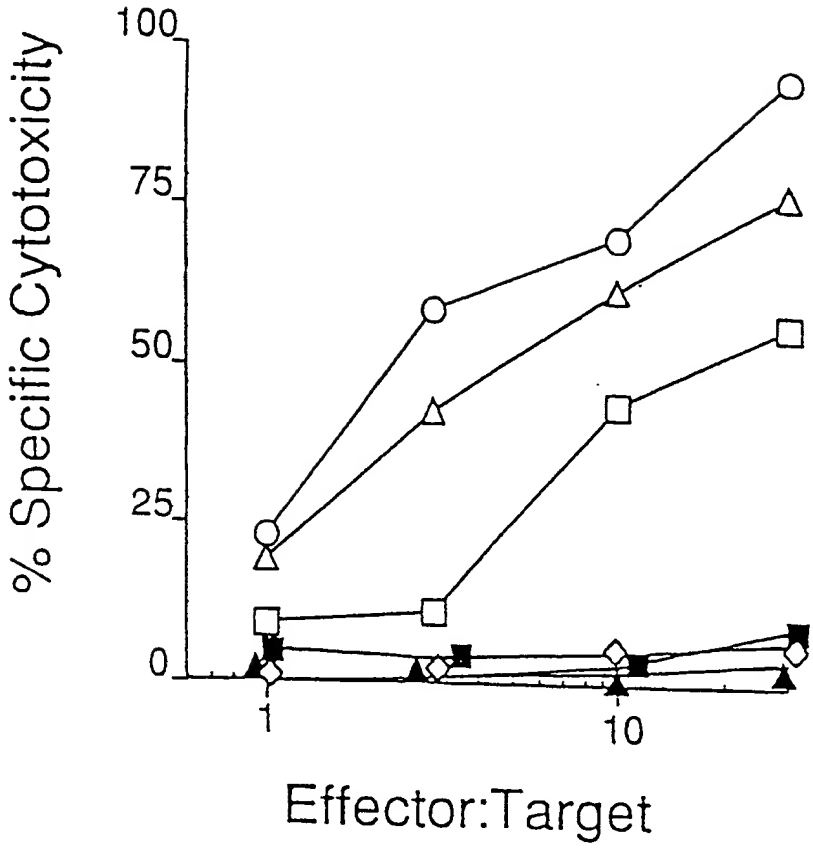

% Specific Lysis
100
75
50
25
0
A
1
10
60
30
20
10
0
B
1
10
60
Effectors : Target

FIGURE 5


100
75
50
25
0
% Specific Cytotoxicity
1
10
Effector:Target

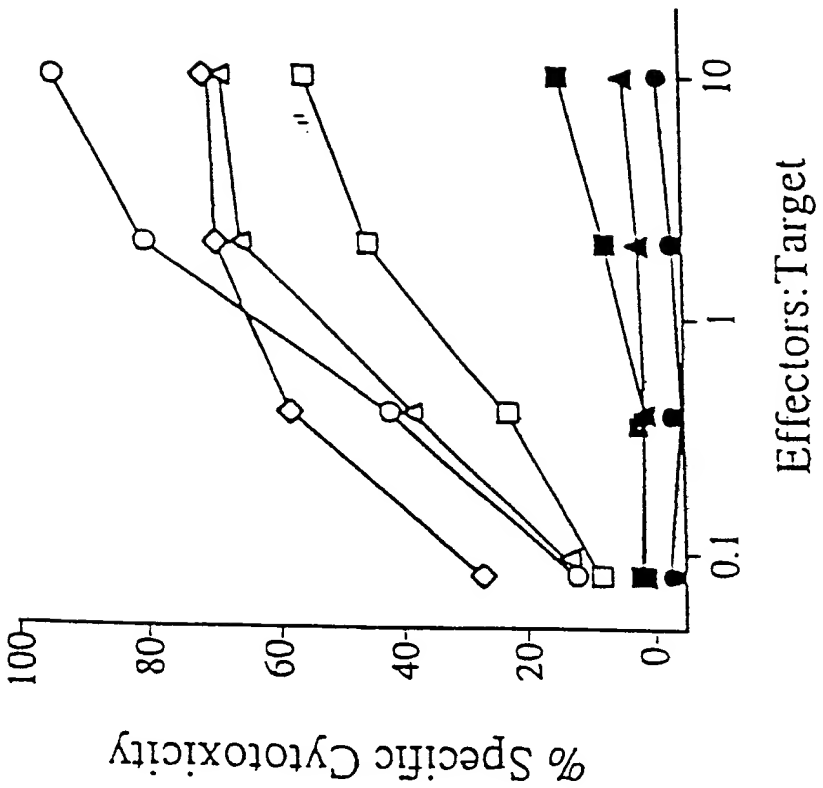

% Specific Cytotoxicity
100
80
60
40
20
0
0.1
1
10
Effectors:Target

FIG. 7

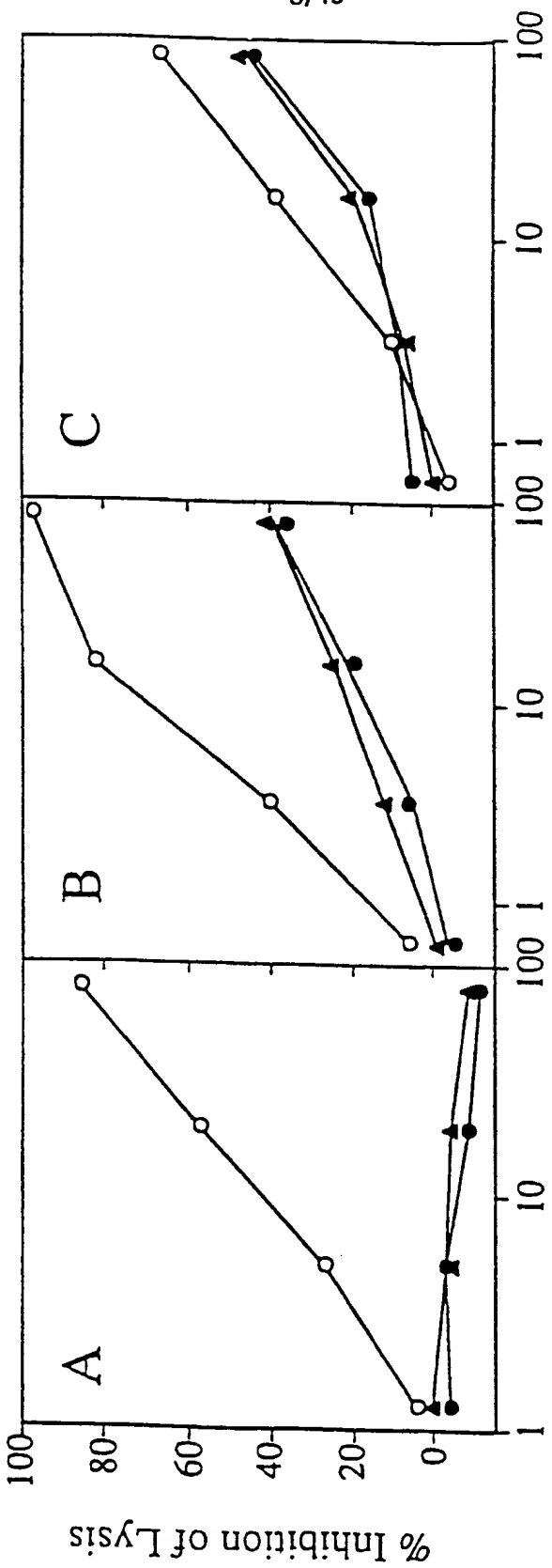
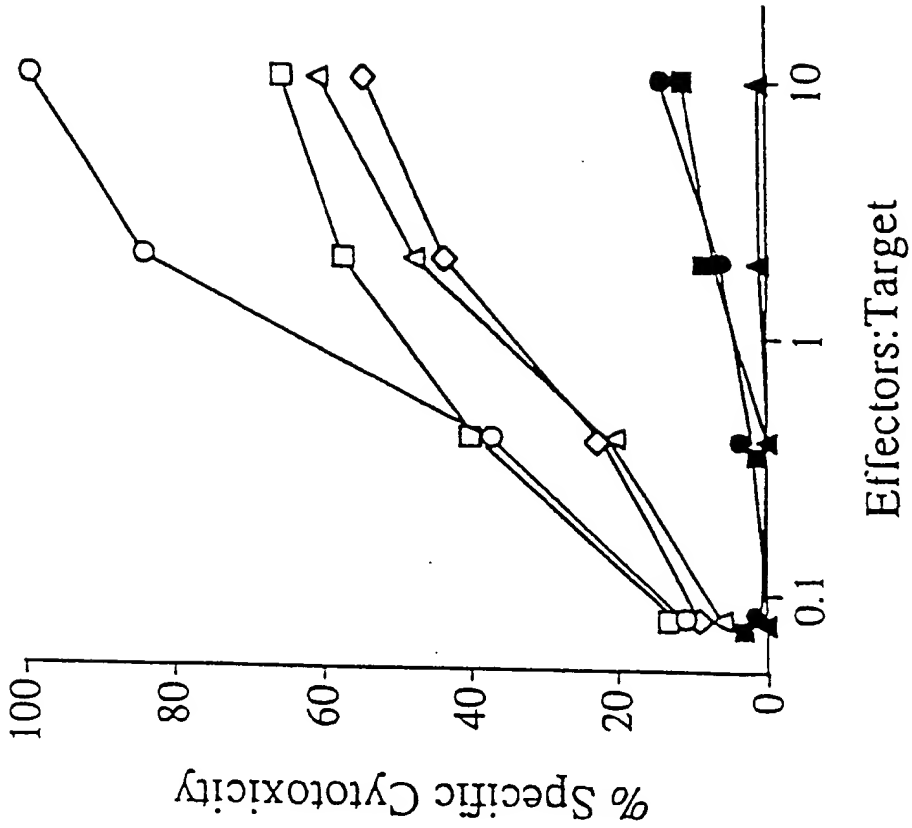


FIG. 8A

FIG. 8B

FIG. 8C

FIG. 9


% Specific Cytotoxicity
100
80
60
40
20
0
0.1
1
10
Effectors:Target

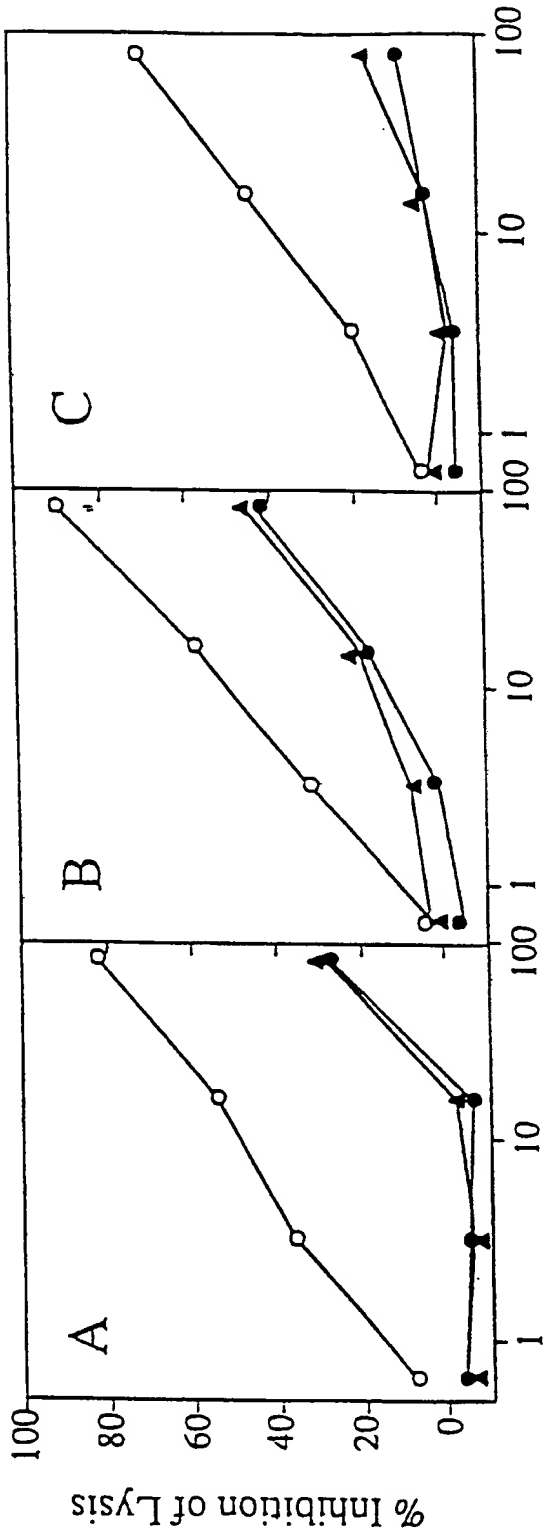


FIG. 10A

FIG. 10B

FIG. 10C

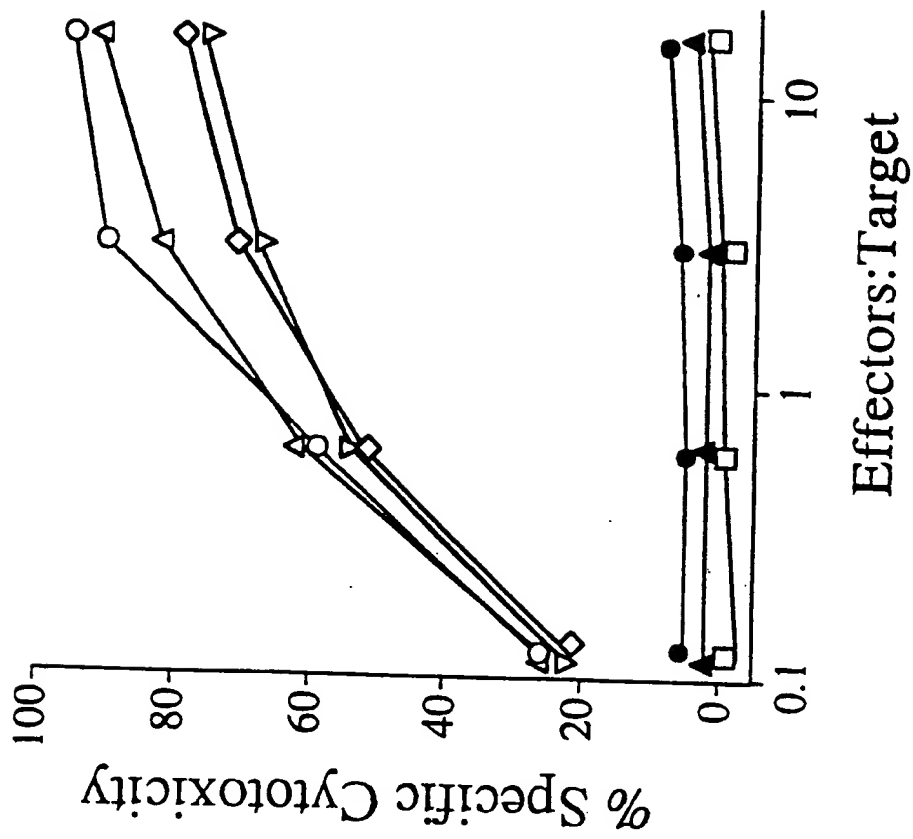

% Specific Cytotoxicity
100
80
60
40
20
0
0.1
1
10
Effectors:Target

FIG. 11

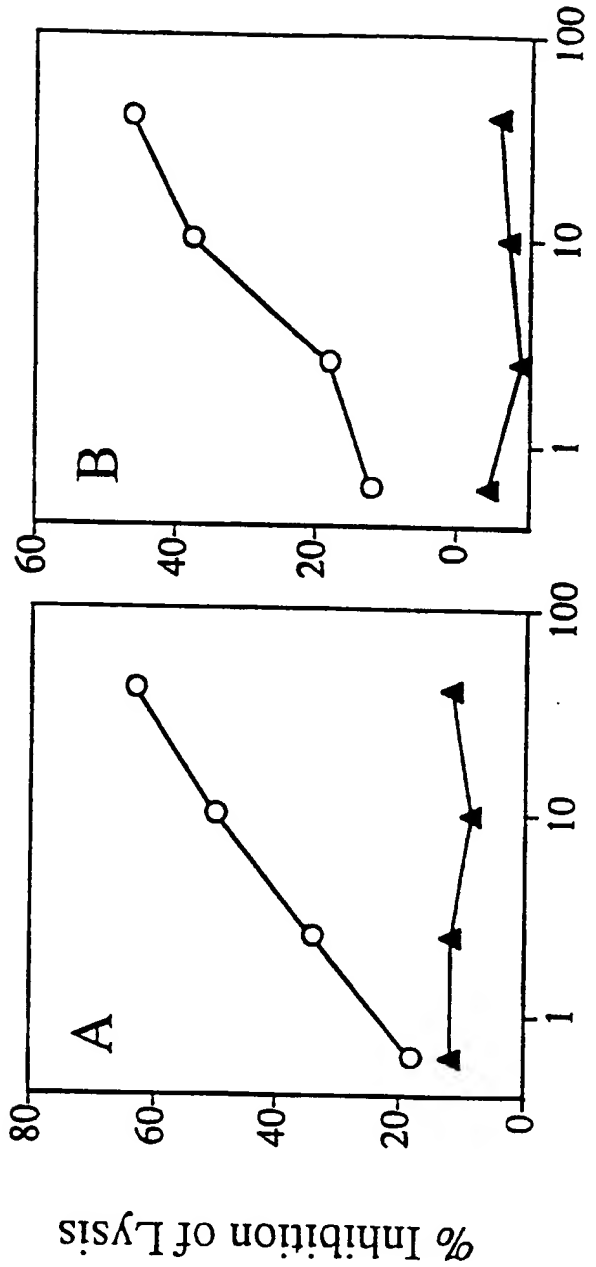

% Inhibition of Lysis
A
B
0
20
40
60
80
1
10
100
Inhibitors: Target

FIG. 12

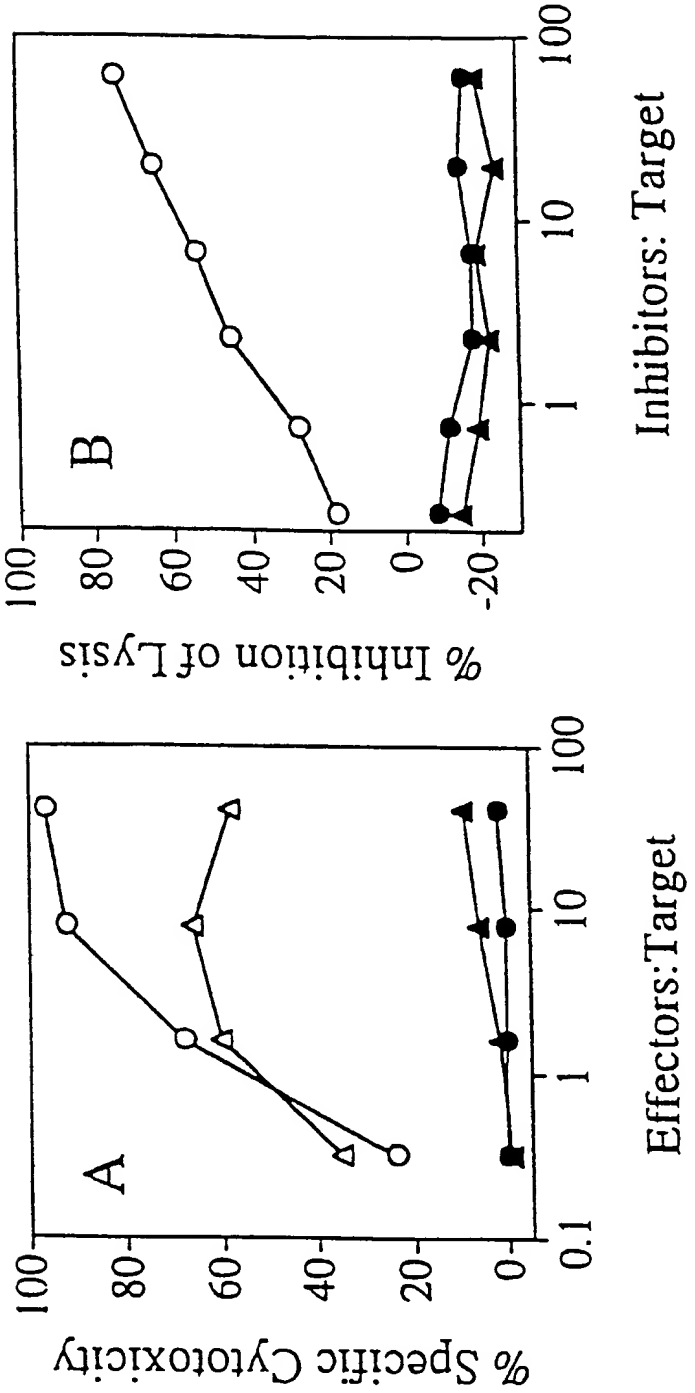


FIG. 13A

FIG. 13B

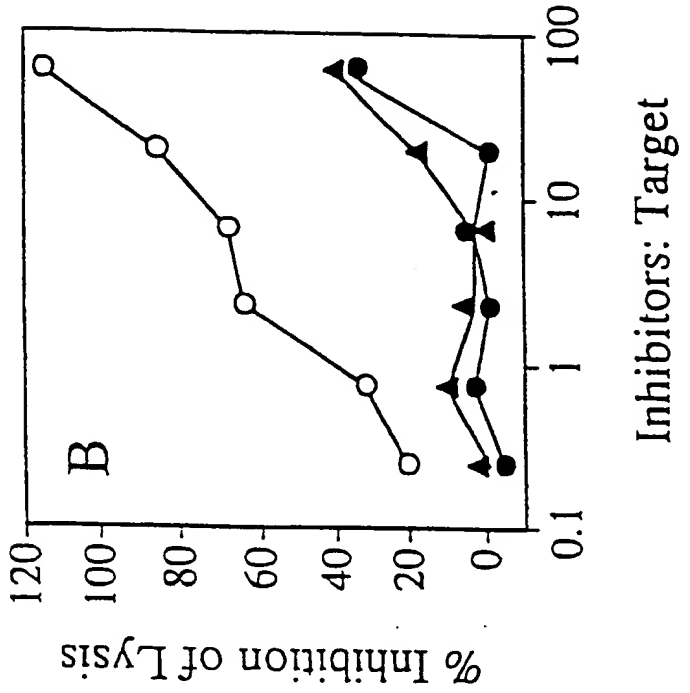


FIG. 14B

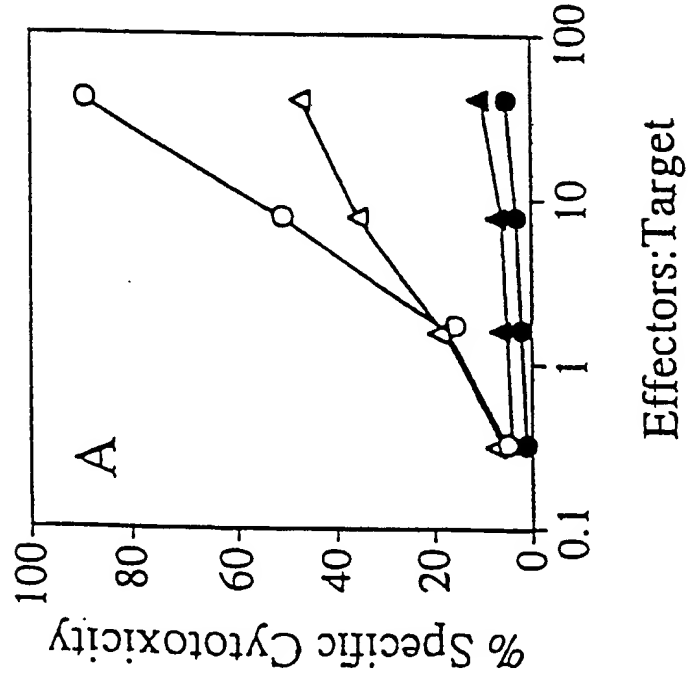


FIG. 14A

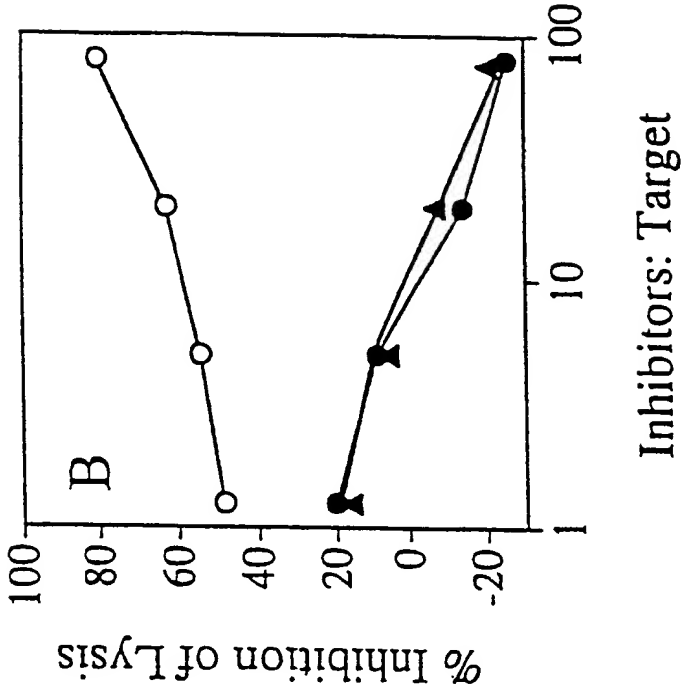


FIG. 15B

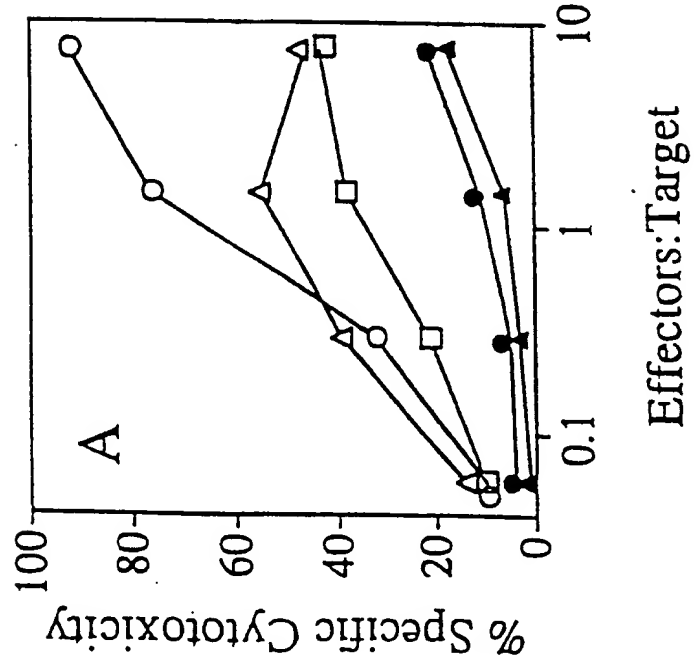


FIG. 15A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01959

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/06, 5/08
US CL :435/372.3, 377, 385, 386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/372.3, 377, 385, 386

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, SCISEARCH, DERWENT WORLD PATENT, EMBASE, BIOSIS, IL-4, Il-10, Il-4, GM-CSF , antigen presenting cell, APC, dendritic cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BENDER et al. Improved Methods For The Generation Of Dendritic Cells From Nonproliferating Progenitors In Human Blood. J. of Immunological Methods. 1996. Vol. 196, pages 121-135, see entire article.	1-19
Y	WENTWORTH et al. In Vitro Induction Of Primary, Antigen-Specific CTL From Human Peripheral Blood Mononuclear Cells Stimulated With Synthetic Peptides. Molecular Immunology. 1995. Vol. 32. No. 9, pages 603-612, see entire article.	1-2, 4-6, 8, 9-12, 14, 15-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 APRIL 1998	Date of mailing of the international search report 29 MAY 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARTHA LUBET Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01959

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BUELENS et al. Interleukin-10 Differentially Regulates B7-1 (CD80) and B7-2 (CD86) Expression On Human Peripheral Blood Dendritic Cells. Eur. J. Immunol. 1995. Vol. 25, pages 2668-2672, see entire article.	1-2, 4-5, 7-12, 14, 15, 17-19,
Y	WO 94/03205 A1 (CYTEL CORPORATION). 17 February 1994, see entire document.	1-19
Y	STEINMAN, RM. Dendritic Cells And Immune-Based Therapies. Experimental Hematology. 1996. Vol. 24. pages 859-862, see entire article.	1-19
Y	MACATONIA et al. Primary Proliferatiave And Cytotoxic T-Cell Responses To HIV Induced In Vitro By Human Dendritic Cells. Immunology. 1991. Vol. 74. pages 399-406, see entire article.	1-19
Y	ROMAINI et al. Generation Of Mature Dendritic Cells From Human Blood An Improved Method With Special Regard To Clinical Applicability. J. of Immunological Methods. 1996. Vol. 196, pages 137-151, see entire article.	1-19